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Pro-inflammatory GPR75 and anti-apoptotic phospholipase signaling pathways contribute to the ameliorating effect of soluble epoxide hydrolase inhibition on chronic experimental autoimmune encephalomyelitis in mice

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ABSTRACT

Soluble epoxide hydrolase (sEH) inhibition has currently emerged as a therapeutic target in the treatment of various neuroinflammatory neurodegenerative diseases, including multiple sclerosis. Previously, we reported that treatment of mice with an sEH-selective inhibitor, 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy) phenyl]urea; TPPU), ameliorated chronic experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein $_{35-55}$ peptide immunization followed by injection of pertussis toxin to mice via regulating pro-inflammatory and anti-inflammatory pathways in the central nervous system. This study tested the hypothesis that the pro-inflammatory G protein-coupled receptor (GPR) 75 and anti-apoptotic phospholipase C (PLC) signaling pathways also contribute to the ameliorating effect of TPPU on chronic EAE. Brains and spinal cords of phosphate-buffered saline-, dimethyl sulfoxide-, or TPPU (3 mg/kg)-treated mice were used for the measurement of sEH, GPR75, $G\alpha_{q/11}$, activator protein (AP)-1, PLC β4, phosphoinositide 3-kinase (PI3K) p85α, Akt1, mitogen-activated protein kinase kinase (MEK) 1/2, extracellular signal-regulated kinase (ERK) 1/2, cyclic adenosine monophosphate-response element-binding protein (CREB) 1, B-cell lymphoma (Bcl)-2, semaphorin (SEMA) 3A, and myelin proteolipid protein (PLP) expression and/or activity by using the immunoblotting method. Expression of sEH, GPR75, $G\alpha_{q/11}$, c-jun, phosphorylated c-Jun, and SEMA3A was lower, while PLCβ4, phosphorylated PI3K p85α, phosphorylated Akt1, phosphorylated MEK1/2, phosphorylated ERK1/2, phosphorylated CREB1, Bcl-2, and myelin PLP expression was higher in the tissues of TPPU (3 mg/kg)-treated mice as compared with the EAE and vehicle control groups. Inhibition of sEH by TPPU ameliorates chronic EAE through suppressing pro-inflammatory GPR75/$G\alpha_{q/11}$/AP-1 pathway and reducing expression of the remyelination inhibitor, SEMA3A, as well as increasing anti-apoptotic PLC/PI3K/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 pathway activity and myelin PLP expression.

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Introduction

Epoxyeicosatrienoic acids (EETs) are synthesized endogenously during arachidonic acid metabolism mostly by cytochrome P450 (CYP) 2C and CYP2J epoxygenases (1). The enzyme soluble epoxide hydrolase (sEH) degrades EETs enzymatically to inactive dihydroxyeicosatrienoic acids (DHETs) (1). EETs or pharmacological inhibition of sEH are reported to have protective effects in the diseases of the central nervous system (CNS) by regulating cerebral blood flow, angiogenesis, and certain signal transduction pathways, including pro-inflammatory and anti-apoptotic mechanisms in the brain (2). In recent years, many investigations have focused on the potential use of especially multitarget sEH inhibitors with higher effectiveness and safety in the treatment of neuroinflammatory and neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and depression (3). On the other hand, only the results of a few studies published to date support the concept that EETs are also involved in the physiopathology of multiple sclerosis (MS), one of the chronic inflammatory demyelinating diseases of the CNS, and sEH-selective inhibitors may represent a promising therapeutic approach for reducing neuroinflammation and accelerating anti-inflammatory responses as demonstrated in its animal model, experimental autoimmune encephalomyelitis (EAE) (4,5).

The classical mitogen-activated protein kinase (MAPK) family consists of three subfamilies: (1) extracellular signal-regulated kinase (ERK) (ERK1 and ERK2), (2) c-Jun-N-terminal kinase (JNK) (JNK1, JNK2, and JNK3), and (3) p38 MAPK (α, β, δ, and γ) (6). The kinases are involved in modulating diverse physiological and physiopathological processes, including cell growth, division, differentiation, motility, metabolism, viability, and inflammation (7). However, conflicting roles of MAPKs in some physiological processes such as apoptosis depending on the cell type and the stimulus have been described. For

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instance, it has been reported that MAPKs, especially p38 MAPK and JNKs activated in response to various biological, chemical, and physical stress stimuli, exert an apoptotic effect through pro-apoptotic proteins (such as caspase-8, caspase-9, Bim and Bcl-2 family member BAD, Hid, and signal transducer and activator of transcription 3/5) (8,9). On the other hand, some MAPKs such as ERK1/2 activated by phosphorylated mitogen-activated protein kinase (MEK) 1/2 have also been shown to exert an anti-apoptotic effect through anti-apoptotic proteins (e.g., Mcl-1, B-cell lymphoma [Bcl]-2, Bcl-XL, IEX-1, c-Flip, and cyclic adenosine monophosphate response element-binding protein [cyclic adenosine monophosphate-response element-binding protein; CREB]) (9,10). Moreover, increased gene expression of mainly inducible nitric oxide (NO) synthase (iNOS), cyclooxygenase (COX) 2, and pro-inflammatory cytokines induced by transcription factors such as activated protein (activated protein; AP)-1 and nuclear factor (NF)-κB due to the changes in the activities of various MAPKs such as JNK, ERK1/2, and p38 MAPK results in the cell/tissue injury, inflammation, neurodegeneration, and apoptosis has been reported to play a role in the pathogenesis of the certain CNS diseases, including MS (11,12). Although only a few studies are reporting the involvement of MAPKs, including JNK, p38 MAPK, and ERK1/2, in neuronal apoptosis during EAE (13-15), further investigations are needed to demonstrate the contribution of the anti-apoptotic MEK1/2/ERK1/2 signaling pathway in the CNS to the pathogenesis of MS.

G protein-coupled receptors (GPRs) are a large family of mammalian seven-transmembrane proteins which are expressed in numerous tissues and considered prominent therapeutic drug targets (16,17). GPR75, one of the orphan GPRs, is expressed in various tissues, including the brain and spinal cord as well as renal, cerebral, aortic, mesenteric, and pulmonary endothelial and/or vascular smooth muscle cells in addition to the brain, kidney, liver, testis, skeletal muscle, and spleen tissues of rodents in addition to human and mouse-human islets (18-22). It can be activated by various stimuli such as proinflammatory cytokines and eicosanoids which play a substantial role in the pathogenesis of certain disorders not only limited to vascular and endothelial inflammation but also neuroinflammation (23). GPR75 can activate various signal transduction pathways via the $G\alpha_{q/11}$ subunit, which can be stimulated by cytokines that play an important role in inflammation (17,23,24). For instance, as an endogenous ligand of GPR75, regulated on activation, normal T cell expressed and secreted is reported to stimulate GPR75, cause activation of anti-apoptotic via the phospholipase C (PLC)/phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway, and prevents amyloid-β-induced cell death in the hippocampal cell line (19). Moreover, recent studies have demonstrated that GPR75, $G\alpha_{q/11}$, and AP-1 have participated in the physiopathology of MS and EAE (25-27). Hence, the data give a reason to the hypothesis that increased activity of the GPR75/$G\alpha_{q/11}$/AP-1 signaling pathway resulting in the formation of pro-inflammatory cytokines plays a key role in the pathogenesis of MS and associated symptoms.

According to the findings of our previous study (4), increased levels of endogenously produced anti-inflammatory EETs associated with the expression of peroxisome proliferator-activated receptor (PPAR) α/β/γ and caspase-recruitment and activation domain-containing nucleotide-binding oligomerization domain-like receptor (NLRC) 3 proteins in addition to the suppressed activity of inflammatory toll-like receptor (TLR) 4/myeloid differentiation factor (MyD) 88/NF-κB signaling pathway, NLRC4/apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC)/pro-caspase-1 inflammasome, caspase-11 inflammasome, and nicotinamide adenine dinucleotide phosphate oxidase (NOX) that are responsible for the production of inflammatory mediators may involve in the protective effect of selective sEH inhibitor, 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl]urea; TPPU), against EAE-induced inflammation and demyelination in the CNS of mice. As a continuation of our previous work, we tested the hypothesis that reduced activity of pro-inflammatory GPR75/$G\alpha_{q/11}$/AP-1 signaling pathway and expression of remyelination inhibitor, semaphorin (SEMA) 3A, as well as increased activity of antiapoptotic PLC/PI3K/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 signaling pathway and expression of the main protein of the CNS myelin membrane, myelin proteolipid protein (PLP), also contribute to the ameliorating effect of sEH inhibition on signs of the disease seen in EAE.

Materials and Methods

Animals and tissues

In our previous study, the in vivo experimental protocol was approved by the Mersin University Experimental Animals Local Ethics Committee (Approval Date: June 11, 2018; Decision Number: 2018/29). The in vivo and ex vivo experiments were carried out from September to December 2019. The animals used in these experiments were housed under standard conditions with free access to food and water and a 12 h light-dark cycle at the Research Center of Experimental Animals, Mersin University, Mersin, Turkey. All in vivo experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the American Veterinary Medical Association Guidelines for the Euthanasia of Animals 2013 Edition. The isolated brain and spinal cord tissues of female C57BL/6 mice (20 to 40 g; n=22) obtained from our previous study (4) were used in these experiments. The present study was carried out from March to October 2021.

Induction of chronic EAE

In our previous study, chronic EAE was induced using myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide immunization followed by injection of pertussis toxin (PT) into female C57BL/6 mice (4). Briefly, the mice were immunized with an injection of MOG_{35-55} peptide (M4939; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in CFA (F5881; Sigma) (200 µg/mouse; 0.1 mL/mouse) subcutaneously on day 0. Additionally, PT (19546; Cayman Chemical Co., Ann Arbor, MI, USA) in PBS (400 ng/mouse; 0.1 mL/mouse) was injected into the mice intraperitoneally on the day of immunization. The animals were also injected with PT 2 days later and scored for EAE signs daily. Upon reaching a disease score of ≥ 2 or ≤ 3 on day 17, mice were injected with intraperitoneal PBS (10010023; Thermo Fisher Sci., Waltham, MA, USA) (0.1 mL/mouse), DMSO (60153; A1584; Applichem GmbH,

Darmstadt, Germany) (0.1 mL/ mouse), or TPPU at 1, 3, or 10 mg/kg doses (in 7% DMSO; 0.1 mL/mouse) daily until the end of the study (4). When PBS-treated mice were scored as a ~ 3 on seven consecutive days, all animals were euthanized, and brain and spinal cord tissues were isolated and then stored at -80°C. The frozen tissues of EAE mice injected with TPPU at the effective dose, of 3 mg/kg, were used in the present study as well.

Immunoblotting

The immunoblotting technique was used according to the protocol as described in detail previously (4,22,28). In brief, tissue homogenates (30 µg of protein) were subjected to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Nitrocellulose membranes containing transferred proteins from the gel were blocked with non-fat dry milk in Tris-buffered saline and then incubated with specific antibodies against (1) sEH (13560; Cayman), (2) GPR75 (GTX55193; GeneTex, Hsinchu City, Taiwan), (3) $G\alpha_{q/11}$ (sc-515689; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (4) c-Jun (sc-1694; Santa Cruz), (5) phosphorylated c-Jun (p-c-Jun) (sc-16312; Santa Cruz), (6) PLC β4 (sc-16631; Santa Cruz); (7) PI3K p85α (OAAF05915; Aviva Systems Biology, San Diego, CA, USA), (8) phosphorylated PI3K p85α (p-PI3K p85α) (OAEC00301; Aviva Systems Biology), (9) Akt1 (sc-271149; Santa Cruz), (10) phosphorylated Akt1 (p-Akt1) (sc-52940; Santa Cruz), (11) MEK1/2 (sc-81504; Santa Cruz), (12) phosphorylated MEK1/2 (p-MEK1/2) (sc-81503; Santa Cruz), (13) ERK1/2 (sc-514302; Santa Cruz), (14) phosphorylated ERK1/2 (p-ERK1/2) (sc-136521; Santa Cruz), (15) CREB1 (sc-271; Santa Cruz), (16) phosphorylated CREB1 (p-CREB1) (sc-81486; Santa Cruz), (17) Bcl-2 (sc-7382; Santa Cruz), (18) myelin PLP (bs-11093R-HRP; Bioss Antibodies Inc., Woburn, MA, USA), and (19) SEMA3A (sc-74554; Santa Cruz) in bovine serum albumin overnight at 4°C. The membranes were then incubated with appropriate secondary antibodies (sheep anti-mouse [RPN4201; Amersham Life Sciences, Cleveland, OH, USA] or goat anti-rabbit IgG-horseradish peroxidase [RPN4301; Amersham]) in bovine serum albumin at room temperature for 1 h. The immune complexes were detected by Enhanced Chemiluminescence Prime Western Blotting Detection Reagent (RPN2232; Amersham) according to the manufacturer's instructions. The photos of the immunoreactive bands were captured with a gel-imaging system (EC3-CHEMI HR imaging system; Ultra-Violet Products, UVP, Cambridge, UK). To determine the relative immunoreactive densities of bands, Image J densitometry analysis software (Image J 1.46r, Wayne Rasband, National Institute of Health, Bethesda, MD, USA) was used. The membranes were reused for anti-β-tubulin antibody (sc-5274; Santa Cruz) as a loading control. The ratio of each band/β-tubulin was taken into account for the expression levels of specific proteins.

Statistical analysis

The results are expressed as means ± standard error of the mean (SEM). Since the data were normally distributed, parametric statistical analysis for the comparison of two groups of data was conducted using the Student's *t*-test. Statistical analysis was performed using GraphPad Prism version 7.04 for Windows (GraphPad Software, San Diego California USA; http://www.graphpad.com). $P<0.05$ was considered to indicate a statistically significant difference.

Results

MOG_{35-55} peptide/PT-induced sEH expression was decreased by TPPU treatment

Previously, we demonstrated that MOG_{35-55} peptide/PT-induced levels of 14,15-DHET, the biologically less active metabolite of 14,15-EET, were reduced in the brain and spinal cord tissues of TPPU-treated mice (4). In the present study, we tested whether TPPU also affects protein expression of sEH in the CNS of EAE-mice, the brain and spinal cord tissue samples of phosphate-buffered saline (PBS)-, dimethyl sulfoxide (DMSO)-, or TPPU-treated EAE mice were immunoblotted with a specific sEH antibody. Reduced expression of sEH was observed in the brains (Figure 1a) and spinal cords (Figure 1b) of TPPU-treated mice compared with the EAE and vehicle control groups ($P<0.05$). Expression of sEH in the tissues of DMSO-treated mice did not differ from control group values ($P>0.05$).

MOG_{35-55} peptide/PT-induced GPR75/$G\alpha_{q/11}$/AP-1 signaling pathway activity was decreased by TPPU treatment

The orphan GPRs, including GPR75, $G\alpha_{q/11}$, and AP-1 have been reported to contribute to the pathogenesis of MS/EAE (13,16,17). To test whether TPPU affects the activity of pro-inflammatory GPR75/$G\alpha_{q/11}$/AP-1 signaling pathway in the CNS of EAE-mice, the brain and spinal cord tissue samples of PBS-, DMSO-, or TPPU-treated EAE mice were immunoblotted with specific antibodies for GPR75, $G\alpha_{q/11}$, c-Jun (a key subunit of AP-1), and p-c-Jun (at Ser^{63}/Ser^{73}). Reduced expression of GPR75, $G\alpha_{q/11}$, c-Jun, and p-c-Jun was observed in the brains (Figure 2a-c) and spinal cords (Figure 2d-f) of TPPU-treated mice compared with the EAE and vehicle control groups ($P<0.05$). Expression of unphosphorylated and phosphorylated proteins in the tissues of DMSO-treated mice did not differ from control group values ($P>0.05$).

MOG_{35-55} peptide/PT-induced PLC/PI3K/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 signaling pathway activity was increased by TPPU treatment

There is accumulating evidence suggesting that PLC/

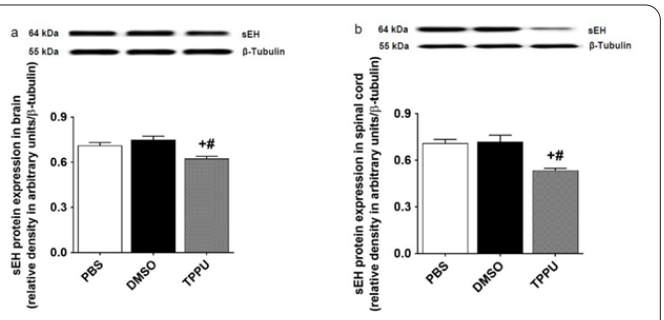

Figure 1. TPPU decreased the sEH protein expression induced by MOG_{35-55} peptide/PT in the (a) brain and (b) spinal cord of PBS-, DMSO-, or TPPU-injected mice. Expression of sEH protein in the tissues was measured by immunoblotting technique. Data are expressed as means ± SEM of 3-4 animals. $^{+}$ $P<0.05$ versus PBS-injected mice (EAE control group); $^{\#}$ $P<0.05$ versus DMSO-injected mice (vehicle control group).

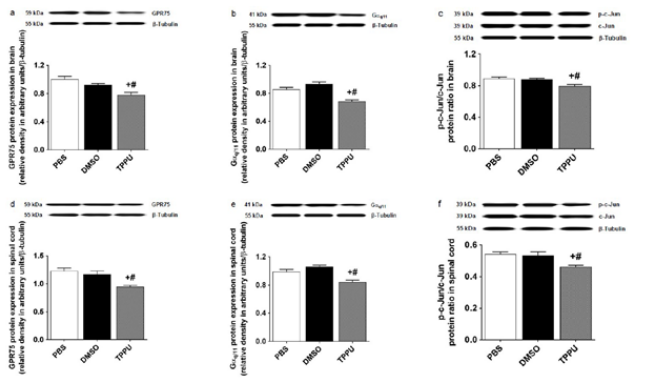

Figure 2. TPPU decreased the GPR75, G$\alpha_{q/11}$, and c-Jun expression and/or activity induced by MOG$_{35-55}$ peptide/PT in the (a, b, and c) brain and (d, e, and f) spinal cord of PBS-, DMSO-, or TPPU-injected mice. The expression of unphosphorylated and phosphorylated proteins for GPR75, G$\alpha_{q/11}$, c-Jun, and p-c-Jun in tissues was measured by immunoblotting technique. Data are expressed as means ± SEM of 3-4 animals. $^{+}$ P<0.05 versus PBS-injected mice (EAE control group); $^{\#}$ P<0.05 versus DMSO-injected mice (vehicle control group).

PI3K/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 signaling pathway plays a significant role in MS pathology as demonstrated in its experimental models (29-41). To investigate the effect of TPPU on the activity of the signaling pathway in the CNS of EAE mice, the brain and spinal cord tissue samples of PBS-, DMSO-, or TPPU-treated EAE mice were immunoblotted with specific antibodies for PLC β4, PI3K p85α, p-PI3K p85α (at Tyr^{467}), Akt1, p-Akt1 (at Ser^{473}), MEK1/2, p-MEK1/2 (at Ser^{218}/Ser^{222} for MEK1 and Ser^{222}/Ser^{226} for MEK2), ERK1/2, p-ERK1/2 (at Thr^{203}/Tyr^{205} for ERK1 and (at Thr^{183}/Tyr^{185} for ERK2), CREB1, p-CREB1 (at Ser^{133}), and Bcl-2. Enhanced expression of PLC β4, p-PI3K p85α, p-Akt1, p-MEK1/2, p-ERK1/2, p-CREB1, and Bcl-2 was observed in the brains (Figure 3a-g) and spinal cords (Figure 3h-n) of TPPU-treated mice compared with the EAE and vehicle control groups (P<0.05). Expression of unphosphorylated and phosphorylated proteins in the tissues of DMSO-treated mice did not differ from control group values (P>0.05).

MOG$_{35-55}$ peptide/PT-induced SEMA3A expression was decreased by TPPU treatment

In recent years, some members of the semaphorin family, including SEMA3A, one of the remyelination inhibitors, have received major attention in the pathogenesis of MS clinically and experimentally (33,42). To investigate the effect of TPPU on SEMA3A expression in the CNS of EAE mice, the brain and spinal cord tissue samples of PBS-, DMSO-, or TPPU-treated EAE mice were immunoblotted with a specific SEMA3A antibody. Reduced expression of SEMA3A was observed in the brains (Figure 4a) and spinal cords (Figure 4b) of TPPU-treated mice compared with the EAE and vehicle control groups (P<0.05). Expression of SEMA3A in the tissues of DMSO-treated mice did not differ from control group values (P>0.05).

MOG$_{35-55}$ peptide/PT-induced Myelin PLP expression was increased by TPPU treatment

Loss of myelin proteins such as PLP, in the CNS has also been implicated in the pathogenesis of MS (43) and experimental EAE models (33). To investigate the effect of TPPU on myelin PLP expression in the CNS of EAE mice, the brain and spinal cord tissue samples of PBS-, DMSO-, or TPPU-treated EAE mice were immunoblotted with a specific myelin PLP antibody. Enhanced expression of myelin PLP was observed in the brains (Figure 5a) and spinal cords (Figure 5b) of TPPU-treated mice compared with the EAE and vehicle control groups (P<0.05). Expression of myelin PLP in the tissues of DMSO-treated mice did not differ from control group values (P>0.05).

Discussion

The findings of the present study indicate that the sEH-selective inhibitor, TPPU, ameliorates the symptoms of MOG$_{35-55}$ peptide/PT-induced chronic EAE in mice by (1) suppressing pro-inflammatory GPR75/G$\alpha_{q/11}$/AP-1 signaling pathway activity, (2) enhancing anti-apoptotic PLC/PI3K/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 signaling pathway activity, (3) inhibiting demyelination, and (4) promoting remyelination in the CNS. The results indicate that anti-inflammatory EETs are involved in the pathogenesis of MS. Moreover, increasing endogenous EET levels

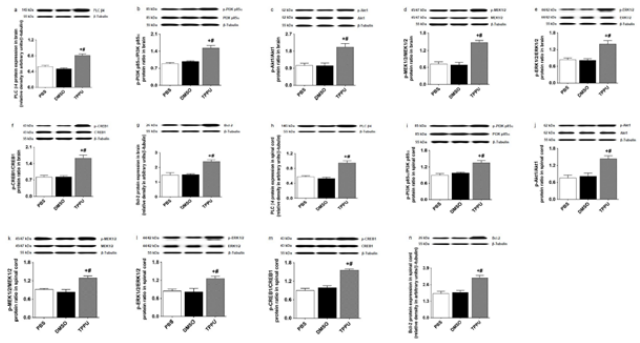

Figure 3. TPPU increased the PLC β4, PI3K p85α, Akt1, MEK1/2, ERK1/2, CREB1, and Bcl-2 expression and/or activity induced by MOG$_{35-55}$ peptide/PT in the (a, b, c, d, e, f, and g) brain and (h, i, j, k, l, m, and n) spinal cord of PBS-, DMSO-, or TPPU-injected mice. The expression of unphosphorylated and phosphorylated proteins for PLC β4, PI3K p85α, Akt1, MEK1/2, ERK1/2, CREB1, and Bcl-2 in tissues was measured by immunoblotting technique. Data are expressed as means ± SEM of 3-4 animals. $^{+}$ P<0.05 versus PBS-injected mice (EAE control group); $^{\#}$ P<0.05 versus DMSO-injected mice (vehicle control group).

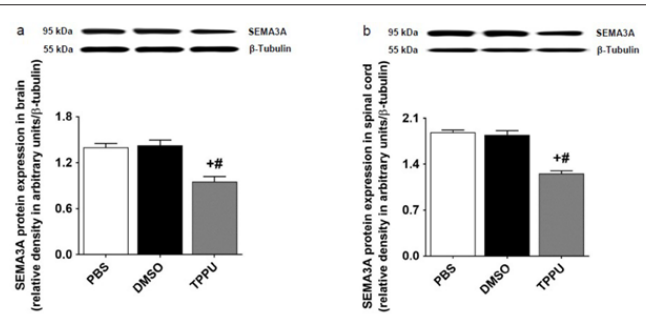

Figure 4. TPPU decreased the SEMA3A protein expression induced by MOG$_{35-55}$ peptide/PT in the (a) brain and (b) spinal cord of PBS-, DMSO-, or TPPU-injected mice. Expression of SEMA3A protein in tissues was measured by immunoblotting technique. Data are expressed as means ± SEM of 3-4 animals. $^{+}$ P<0.05 versus PBS-injected mice (EAE control group); $^{\#}$ P<0.05 versus DMSO-injected mice (vehicle control group).

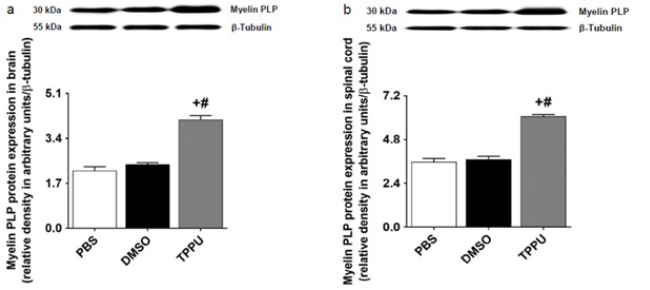

Figure 5. TPPU increased the myelin PLP protein expression induced by MOG_{35-55} peptide/PT in the (a) brain and (b) spinal cord of PBS-, DMSO-, or TPPU-injected mice. Expression of myelin PLP in tissues was measured by immunoblotting technique. Data are expressed as means ± SEM of 3-4 animals. $^{+}$ P<0.05 versus PBS-injected mice (EAE control group); $^{\#}$ P<0.05 versus DMSO-injected mice (vehicle control group).

by suppressing both the expression and activity of sEH pharmacologically protects against not only inflammation but also apoptosis and demyelination in the animal model of MS.

EAE is the most widely used immune-mediated *in vivo* model of MS in preclinical studies (44). Induction of EAE can be achieved in various species by immunization using a myelin antigen (e.g., myelin proteins MOG, PLP, myelin basic protein [MBP] or fragments of these proteins) in an adjuvant (e.g., complete Freund's adjuvant; CFA) (active EAE) or by the adoptive transfer of encephalitogenic T cells (passive EAE) (44). In these models, myelin autoimmune responses are taken into account for not only demyelination and neurodegeneration but also inflammation in response to an autoimmune disease mediated by T-helper cells in the CNS (45). The clinical manifestations of EAE are attenuated or prevented by treatment with specific drug candidates administrated before, at, or after the peak of the disease, and the pharmacological approaches have preventive effects as demonstrated by protecting demyelination and injury of the myelin sheath or oligodendrocyte cell body (44). Furthermore, there are also studies investigating the effects of various agents on the expression of MOG (46-48), MBP (46,49,50), myelin-associated oligodendrocyte basic protein (MOBP) (46), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (48-51), PLP (49), and SEMA3A (51) proteins that have regulatory roles in remyelination in the brain and/or spinal cord tissues in chronic EAE induced by MOG_{35-55} peptide/PT in rodents. In studies using this model, it has been shown that the expression of MOG, MBP, MOBP, and CNPase, which is measured as a marker of maturation of oligodendrocytes and remyelination, decreases in the chronic phase of EAE (46,47,49,50). In addition, it has been reported that although MBP and CNPase expressions do not change in the acute and chronic stages of EAE, PLP expression decreases, while SEMA3A expression, which is one of the remyelination inhibitors, increases (42,49).

There are studies investigating the effects of TPPU, a selective sEH inhibitor (52-59) on the expression and/or activity of sEH, PI3K, Akt, ERK1/2, CREB, and Bcl-2 under *in vitro* and *in vivo* models of various pathological conditions (53-56). In addition, GPR75, FLC, PI3K, Akt, MAPK, ERK1/2, and CREB have been reported to contribute to the pathogenesis of MS (19,25,29-32,34-41,57,58). Data from a study in which we previously investigated the effect of sEH inhibition on pro-inflammatory and anti-inflammatory pathways in chronic EAE induced by MOG_{35-55} peptide/PT in mice (4) demonstrated that the protective effects of TPPU against EAE-induced inflammation were also associated with increased expression of PPARα/β/ and NLRC3 proteins, as well as decreased expression of TLR4, MyD88, NF-κB p65, p-NF-κB p65, iNOS/neuronal NOS, COX-2, NLRC4, ASC, caspase-1 p20, IL-1β, caspase-11 p20, NOX subunits ($gp91^{phox}$; a superoxide generating NOX enzyme, and $p47^{phox}$; organizer subunit of $gp91^{phox}$), and nitrotyrosine proteins in the brain and spinal cord tissues as compared with the control groups. Furthermore, reduced levels of 14,15-DHET and IL-1β were also observed in these tissues. These findings suggest that not only suppressing of TLR4/MyD88/NF-κB signaling pathway, which is responsible for the generation of pro-inflammatory mediators such as reactive nitrogen/oxygen species, prostaglandins, and cytokines, NLRC4/ASC/pro-caspase-1 inflammasome, and caspase-11 inflammasome as a result of reducing the inactivation of EETs by inhibition of sEH but also increasing NLRC3 and PPARα/β/γ expression may also contribute to the ameliorating effect of TPPU on the findings of EAE. Considering that TPPU is a dual sEH/p38β kinase inhibitor (59), it is also possible that suppressed p38 MAPK signaling pathway activity may also contribute to the inhibitory effect of TPPU on the NLRC4/ASC/pro-caspase-1 inflammasome. In a recent study by Jonnalagadda et al. (5) performed in chronic EAE induced with MOG_{35-55} peptide/PT in mice, it has been shown that oral administration of TPPU prophylactically at a dose of 10 mg/kg accumulate in the spinal cords, which was correlated with its plasma level, and reduces EAE by reducing neuroinflammation and increasing anti-inflammatory responses without causing a decrease in the number of leukocytes in the blood.

In the current study, we further demonstrated that treatment of EAA mice with TPPU resulted in the decreased expression of sEH, GPR75, $G\alpha_{q/11}$, c-jun, p-c-Jun, and SEMA3A in addition to the enhanced expression of PLC β4, PI3K p85α, p-Akt1, p-MEK1/2, p-ERK1/2, p-CREB1, Bcl-2, and myelin PLP in the brains and spinal cord tissues as compared with the control groups. There is accumulating evidence demonstrating that decreased expression of Bcl-2 (60) and PLP (49) as well as enhanced expression and/or activity of Akt1 (61), ERK1/2 (40,61,62), and SEMA3A (42,49) in the CNS of MOG_{35-55} peptide/PT-induced EAE in rodents. Although the inhibitory effect of TPPU on the enhanced sEH expression and the decrease in $G\alpha_{q/11}$ protein expression in this model is consistent with the findings of previous studies (27,54,55), we have demonstrated for the first time its effect on the expression and/or activity of GPR75, c-Jun, phosphorylated c-Jun, FLC β4, PI3K p85α, MEK1/2, and CREB1 proteins in the CNS of EAE mice. Based on the findings of our previous studies (4) along with a recent study investigating the effect of TPPU on EAE (5), these results suggest that inhibition of pro-inflammatory GPR75/$G\alpha_{q/11}$/AP-1 signaling pathway and expression of SEMA3A, as well as enhanced activity of anti-apoptotic PLC/PI3K/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 signaling pathway and expression of myelin PLP, contribute to the ameliorating effect of TPPU. Collectively, the findings demonstrate that anti-inflammatory EETs play a role in MS pathogenesis, and multiple cellular protective effects occur as a result of increasing

endogenous EET levels when pharmacologically inhibited by sEH.

Although the molecular mechanisms of its beneficial effects on EAE could not be investigated in detail within the scope of this study, TPPU may directly or indirectly alter the expression of myelin PLP and SEMA3A proteins through the activity of not only GPR75/$G\alpha_{q/11}$/AP-1, which is responsible for the pro-inflammatory cytokine (e.g., interleukin-1β,) formation leading to the increased the blood-brain barrier permeability and leucocyte infiltration resulted in neuroinflammation and demyelination but also anti-apoptotic PLC β4/Akt1/MEK1/2/ERK1/2/CREB1/Bcl-2 signaling pathways in the CNS. In addition, it can be asserted that TPPU may exert beneficial effects due to its synergistic effect on signal transduction pathways that play a role in the pathogenesis of chronic EAE. However, additional studies should be done to prove the validity of these hypotheses.

In conclusion, according to the data we obtained in the chronic EAE model of MS, which is frequently used in preclinical drug development studies, especially for use in the treatment of primary progressive MS and secondary progressive MS, selective and potent sEH inhibitors such as TPPU, which can be applied systemically, able to cross the blood-brain barrier and have a long half-life may be useful in the prevention and treatment of CNS-related diseases associated with neuroinflammation, apoptosis, demyelination, and neurodegeneration. Due to the anti-inflammatory, anti-apoptotic, anti-demyelination, and remyelinating properties, it is expected that the results obtained from this study will contribute to the preclinical and clinical studies to be conducted to develop sEH inhibitors such as TPPU as drugs for the prevention and treatment of all types of MS.

Interest conflict

The authors declare no conflict of interest/competing interests.

Author's contribution

BT conceptualized and conceived the research design, analyzed the data, and drafted the manuscript. BT, MFH, SPS, OB, and MTR carried out the experiments. SSF and SS contributed to the finalizing of the manuscript. All authors read and approved the final manuscript.

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