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Short-chain fatty acid receptor GPR41-mediated activation of sympathetic neurons involves synapsin 2b phosphorylation

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ABSTRACT

Synapsins are neuronal phosphoproteins that coat synaptic vesicles and are believed to function in the regulation of neurotransmitter release. The signaling mechanism for short-chain free fatty acid (SCFA)-stimulated NE release was examined using primary-cultured mouse sympathetic cervical ganglion neurons. Pharmacological and knockdown experiments showed that activation of sympathetic neurons by SCFA propionate involves SCFA receptor GPR41 linking to Gβγ-PLCβ3-ERK1/2-synapsin 2 signaling. Further, synapsin 2b directly interacts with activated ERK1/2 and can be phosphorylated on serine when SCFA activates sympathetic neurons.

1. Introduction

The sympathetic nervous system (SNS) plays an essential role in the maintenance of body homeostasis. The SNS can be regulated by various stimuli, including free fatty acids (FFAs) [1–4]. An infusion of the long-chain fatty acid oleate (C18:1) increases blood pressure in rats, and the effect can be abolished by the α_1-adrenoceptor blocker prazosin, which indicates that long-chain fatty acids enhance sympathetic activity [5]. In addition, an infusion of the short-chain fatty acid (SCFA) acetate (C2) causes an increase in blood pressure, heart rate, and energy expenditure in normal humans [6], which suggests that SCFAs activate the SNS. However, the mechanism by which fatty acids induce sympathetic activation is unclear.

Recently, the ligands of several orphan G protein-coupled receptors (GPCRs)—GPR40, GPR41, GPR43, GPR84, GPR119, and GPR120—have been identified, and were found to be FFAs. The agonists of GPR40, GPR84, GPR119 and GPR120 are long- and medium-chain fatty acids [7–11], and those of GPR41 and GPR43 are SCFAs [12–15]. We have recently found that GPR41 is expressed abundantly in sympathetic ganglia and that SCFAs directly activate the SNS via GPR41 at the level of the ganglion [16]. In addition, we found that the SCFA propionate (C3) has the most potent agonistic effect on GPR41, whereas the ketone body β-hydroxybutyrate acts as an antagonist of GPR41 in heterologous expression systems [16]. However, the detailed signaling pathway for GPR41-mediated sympathetic activation remains uncertain. In the present study, we clarified the signaling mechanism for the GPR41-mediated release of NE using primary-cultured mouse sympathetic cervical ganglion (SCG) neurons as a model. The finding provides a new insight into the mechanism of GPR41-mediated sympathetic activation by SCFAs.

2. Materials and methods

2.1. Animals

All experimental procedures involving mice were carried out according to protocols approved by the relevant animal ethics

Abbreviations: SCFA, short-chain fatty acid; GPCR, G protein-coupled receptor; NE, norepinephrine; SNS, sympathetic nervous system; FFAs, free fatty acids; SCG, sympathetic cervical ganglion; P1, postnatal day 1; Dox, doxycycline; [^{3}H]-NE, tritium-labeled norepinephrine; DF, Dulbecco's modified Eagle's medium/nutrient Ham's mixture F-12; KRB, Krebs Ringer bicarbonate buffer; PTX, pertussis toxin; MS, mass spectrometry; IP, immunoprecipitation

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committees. Postnatal day 1 (P1) C57/BL6J mice were purchased from Shimizu Laboratory Supplies Co., Ltd. *Gpr41*$^{-/-}$ mice were generated as previously described [16] and backcrossed 11 times into C57BL/6J background.

2.2. Cell culture

Primary culture of SCG neurons was performed as described previously [16]. For quantitative RT-PCR analysis, cells were incubated at 37 °C for 24 h. For immunoprecipitation, Western blot analysis, and NE release assay, cells were incubated at 37 °C for 96 h before assay. HEK293 cells expressing doxycycline (Dox)-inducible mouse GPR41 is cultured as described previously [16].

2.3. Plasmid construction and transfection

Mouse synapsin 2b expression plasmid was constructed by ligating synapsin 2b complementary DNA into the mammalian expression vector pcDNA3.1 (Invitrogen) with the amino-terminal HA tag. S426A mutant of synapsin 2b was generated using the PrimeSTAR Mutagenesis Basal kit (TaKaRa). The primers used were 5′-GCCCTGGCTCCACAGAGACCTTTAACC-3′ and 5′-CTGTGGAGCCAG GGCAGGAGTCCTGGA-3′. HEK293 cells expressing Dox-inducible mouse GPR41 was transfected using Lipofectamine 2000 (Invitrogen). After 24 h, GPR41 protein expression was induced by adding doxycycline hyclate (10 μg/ml, Sigma), and incubated at 37 °C for 48 h before assay.

2.4. RNA isolation and quantitative RT-PCR

Total RNA was extracted from primary-cultured SCG neurons or P1 SCG using RNeasy Mini Kit (Qiagen). Then cDNA was synthesized and analyzed by quantitative RT-PCR [16–18]. After an initial incubation for 30 s at 95 °C, cDNA was amplified 40 cycles of PCR (95 °C, 5 s, 60 °C, 30 s). Expression was quantified in triplicate. The primers used were 5′-CCTGAATGGCAGAAAACCTG-3′ and 5′-TGAATGGGATGGAGAGGAAG-3′ for *Synapsin1*, 5′-GGGTGTTTGCTCA GATGGTG-3′ and 5′-TCTCTCGGTGATTGGGGTAG-3′ for *Synapsin2*, 5′-GATGAAGCCAAAGCGGAGAC-3′ and 5′-GCACAAAGGAACAGAGG AAGG-3′ for *Synapsin2a*, 5′-TGCTGACTGGACTGTTTCTCC-3′ and 5′-AGTGGCTGTTTCTCCCAACC-3′ for *Synapsin2b*, 5′-TCGATGACG CCCATACAGAC-3′ and 5′-GGTAACATAAGCAGCCAGGTTC-3′ for *Synapsin3*, 5′-CTCAACACGGGAAACCTCAC-3′ and 5′-AGACAAATCGCTCC ACCAAC-3′ for *18S*.

2.5. siRNA transfection

Cells were nucleofected as described [19]. siRNAs used were 5′-UUGAUAAGCAGAUUGGAAGGCUUCA-3′ for *Erk1*[16], 5′-AUAA UACUGCUCCAGGUAUGGGUGG-3′ for *Erk2* [16], 5′-AUUUGAUGA ACUUACUCCCGCGCCG-3′ for *Plcb3* [16], 5′-AAAUGUAGUCUUUG CCAUCUUUGCC-3′ for *Synapsin2* (#1), 5′-UUAAAGGUCUCUGUGG AGACAGGGC-3′ for *Synapsin2* (#2), 5′-AUGACUAGAUCAGUGAUGA GUUGUC-3′ for *Synapsin2* (#3). Stealth RNAi™ siRNA Negative Control LO GC (Invitrogen) was used as a non-specific control.

2.6. Tritium-labeled norepinephrine release assay

Tritium-labeled norepinephrine ([^{3}H]-NE) release assay was performed essentially as described [20]. Cells were labeled with 1.5 μCi/well levo-[ring-2,5,6-^{3}H] norepinephrine (1 mCi/ml; PerkinElmer Life Sciences) in Dulbecco's modified Eagle's medium/nutrient Ham's mixture F-12 (D/F) medium containing 10% FBS for 1 h at 37 °C. For treatments, cells were washed with PBS and preincubated for 30 min at 37 °C in Krebs Ringer bicarbonate buffer (KRB; 136 mM NaCl, 4.7 mM KCl, 10 mM $NaPO_4$, 0.9 mM $MgSO_4$, and 0.9 mM $CaCl_2$, pH 7.4) to which desipramine (0.6 μM) and corticosterone (40 μM) had been added, in the presence or absence of inhibitors; β-hydroxybutyrate (1 mM; Sigma), NF023 (20 μM; Calbiochem), Gallein (10 μM; Calbiochem), Pertussis toxin (PTX; 1 μg/ml; Wako), U73122 (5 μM; Wako), U73343 (5 μM; Wako), and U0126 (10 μM; Promega). Desipramine and corticosterone were added for blockade of the neuronal and extraneuronal uptake of norepinephrine, respectively [21]. The cells were subsequently stimulated by the addition of KRB containing desipramine and corticosterone, with either propionate or Carbachol at indicated concentrations, with or without inhibitors for 15 min at 37 °C.

2.7. Immunoprecipitation and Western blotting

Cells were starved medium without FBS or NGF for 5.5 h at 37 °C and then incubated in medium with or without inhibitors for 30 min at 37 °C. Subsequently, the cells were stimulated with propionate (10 mM) for 10 min with or without inhibitors and harvested as described [22]. The cell lysates immunoprecipitated with anti-synapsin 2 (10 μg/ml; Synaptic Systems), anti-HA (1:50, Roche), as described [23].

Western blot analysis was performed as described [22]. The antibodies used ware anti-β-actin (1:1000; Sigma), anti-PLCβ3 (1:1000; Cell Signaling Technology), anti-total-ERK1/2 (1:1000; Cell Signaling Technology), anti-phospho-ERK1/2 (1:1000; Cell Signaling Technology), anti-Phosphoserine (1:1000; Abcam), anti-synapsin 2 (1 μg/ml), anti-HA (1:1000), anti-rabbit IgG (1:5000; GE Healthcare UK) and anti-mouse IgG (1:5000; GE Healthcare UK).

2.8. Statistical analysis

Values are presented as mean ± SEM. Differences between groups were examined for statistical significance using ANOVA followed by Tukey–Kramer multiple comparison test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. SCFAs increase release of NE from sympathetic neurons via GPR41

First, we investigated the effect of SCFAs on NE release in primary-cultured mouse SCG neurons. The [^{3}H]-NE release assay showed that the nicotinic acetylcholine receptor agonist Carbachol [24] increased NE release (Fig. 1A). Also, propionate stimulated the release of [^{3}H]-NE from SCG neurons in a dose-dependent manner (Fig. 1A). However, β-hydroxybutyrate, a ketone body that was found to have an antagonistic effect on GPR41 [16], did not affect NE release, although it significantly suppressed the propionate-induced release of NE (Fig. 1B). Furthermore, the propionate-induced release of NE was abolished in SCG neurons obtained from *Gpr41*$^{-/-}$ mice (Fig. 1C), although Carbachol-induced release of NE was comparable between wild-type and *Gpr41*$^{-/-}$ SCG neurons (Fig. 1C). These results showed that propionate increases the release of NE from primary-cultured mouse SCG neurons via GPR41.

3.2. Propionate-induced release of NE involves Gβγ, PLCβ3, and ERK1/2

Next, we examined the intracellular signaling pathway for GPR41-mediated release of NE in primary-cultured mouse SCG neurons. Pretreatment with pertussis toxin (PTX) significantly suppressed propionate-induced NE release (Fig. 2A), which indicated the involvement of $G_{i/o}$ (Fig. 2A). Furthermore, the Gβγ inhibitor Gallein significantly suppressed the propionate-induced release of NE, whereas the $G\alpha i_{/o}$ inhibitor NF023 showed no inhibitory effect (Fig. 2A), which indicated the involvement of Gβγ but not $G\alpha i_{/o}$. The PLC inhibitor U73122 and the MEK inhibitor