Neurochemistry International 131 (2019) 104563

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Phospholipase C-related catalytically inactive protein regulates lipopolysaccharide-induced hypothalamic inflammation-mediated anorexia in mice

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ARTICLE INFO

Keywords:
AKT
Anorexia
Hypothalamus
Inflammation
PRIP
STAT3

ABSTRACT

Peripheral lipopolysaccharide (LPS) injection induces systemic inflammation through the activation of the inhibitor of nuclear factor kappa B (NF-κB) kinase (IKK)/NF-κB signaling pathway, which promotes brain dysfunction resulting in conditions including anorexia. LPS-mediated reduction of food intake is associated with activation of NF-κB signaling and phosphorylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) in the hypothalamus. We recently reported phospholipase C-related catalytically inactive protein (PRIP) as a new negative regulator of phosphatidylinositol 3-kinase/AKT signaling. AKT regulates the IKK/NF-κB signaling pathway; therefore, this study aimed to investigate the role of PRIP/AKT signaling in LPS-mediated neuroinflammation-induced anorexia.

PRIP gene (*Prip1* and *Prip2*) knockout (*Prip*-KO) mice intraperitoneally (ip) administered with LPS exhibited increased anorexia responses compared with wild-type (WT) controls. Although few differences were observed between WT and *Prip*-KO mice in LPS-elicited plasma pro-inflammatory cytokine elevation, hypothalamic pro-inflammatory cytokines were significantly upregulated in *Prip*-KO rather than WT mice. Hypothalamic AKT and IKK phosphorylation and IκB degradation were significantly increased in *Prip*-KO rather than WT mice, indicating further promotion of AKT-mediated NF-κB signaling. Consistently, hypothalamic STAT3 was further phosphorylated in *Prip*-KO rather than WT mice. Furthermore, suppressor of cytokine signaling 3 (*Socs3*), a negative feedback regulator for STAT3 signaling, and cyclooxogenase-2 (*Cox2*), a candidate molecule in LPS-induced anorexigenic responses, were upregulated in the hypothalamus in *Prip*-KO rather than WT mice. Pro-inflammatory cytokines were upregulated in hypothalamic microglia isolated from *Prip*-KO rather than WT mice.

Together, these findings indicate that PRIP negatively regulates LPS-induced anorexia caused by pro-inflammatory cytokine expression in the hypothalamus, which is mediated by AKT-activated NF-κB signaling. Importantly, hypothalamic microglia participate in this PRIP-mediated process. Elucidation of PRIP-mediated neuroinflammatory responses may provide novel insights into the pathophysiology of many brain dysfunctions.

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https://doi.org/10.1016/j.neuint.2019.104563
Received 3 March 2019; Received in revised form 9 September 2019; Accepted 2 October 2019
Available online 04 October 2019

Abbreviations

CNS	central nervous system
Cox2	cyclooxygenase-2
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde phosphate dehydrogenase
Iba1	ionized calcium-binding adapter molecule 1
IκB	inhibitor of NF-κB
IKK	inhibitor of NF-κB kinase
IL-1β	interleukin-1 beta
IL-6	interleukin-6
$Ins(1,4,5)P_3$	inositol 1,4,5-trisphosphate
ip	intraperitoneal
JAK	Janus kinase
LPS	lipopolysaccharide
MyD88	myeloid differentiation primary-response protein 88
NF-κB	nuclear factor kappa B
NIK	NF-κB-inducing kinase
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-trisphosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphatidylinositol 3-kinase
PLC	phospholipase C
PLCL1	PLC-like 1
PRIP	PLC-related catalytically inactive protein
Prip-KO	*Prip1* and *Prip2* double knockout
qPCR	quantitative real-time polymerase chain reaction
RT-PCR	reverse-transcription polymerase chain reaction
Socs3	suppressor of cytokine signaling 3
STAT3	signal transducer and activator of transcription 3
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
WT	wild-type

1. Introduction

Infectious diseases induce peripheral inflammation, which can eventually spread to the central nervous system (CNS). This causes neuroinflammation accompanied by upregulation of pro-inflammatory cytokines in the brain. The spread of inflammation leads to brain dysfunction, manifesting in symptoms such as fever, sleep disorders, depression-like behavior, and anorexia (Bluthe et al., 2000; Dantzer et al., 2008; Hart, 1988; Kelley et al., 2003).

Anorexia is a common symptom of infectious diseases (Grunfeld and Feingold, 1992; Hart, 1988). A transient reduction in food intake inhibits pathogen proliferation owing to decreased nutrient availability (Murray and Murray, 1979). However, chronic suppression of food intake induces malnutrition and may impair the host immune system (Grinspoon and Mulligan, 2003). Hence, elucidation of the mechanism underlying infectious disease-induced anorexia is important for improving the nutritional status of affected individuals and quality of life.

Peripheral lipopolysaccharide (LPS) administration in mice is widely used to establish a mouse model of fever and other brain-mediated illness responses. LPS activates toll-like receptor 4 (TLR4)-mediated nuclear factor kappa B (NF-κB) signaling via myeloid differentiation primary-response protein 88 (MyD88)-dependent or independent pathways, resulting in the production of pro-inflammatory cytokines and chemokines (Akira and Takeda, 2004). Peripheral injection of LPS induces the elevation of hypothalamic pro-inflammatory cytokines and anorexia (Jang et al., 2010), whereas the deficiency of MyD88 inhibits LPS-induced anorexia and downregulates hypothalamic pro-inflammatory cytokines (Ogimoto et al., 2006). Hence, peripheral LPS administration-induced elevation of hypothalamic pro-inflammatory responses is essential in LPS-induced anorexia.

NF-κB, a transcription factor, is a regulator of genes involved in inflammation and innate immunity, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). NF-κB is regulated by an inhibitor of NF-κB kinase (IKK) downstream of the LPS-stimulated TLR4/MyD88 pathway (Akira and Takeda, 2004), and inhibition of NF-κB signaling abolishes LPS-induced anorexia (Jang et al., 2010). Appetite is also regulated by signal transducer and activator of transcription 3 (STAT3) activation in the hypothalamus (Vaisse et al., 1996). We previously reported that peripheral LPS administration in mice induces IL-6 expression in the hypothalamus, activating Janus kinase (JAK) via a transmembrane receptor, gp130, and phosphorylating STAT3, leading to anorexia (Yamawaki et al., 2010). These findings suggest that NF-κB and STAT3 activity are fundamentally involved in LPS-induced anorexia.

LPS-stimulated TLR4/MyD88 mediates the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, activating IKK/NF-κB and elevating pro-inflammatory cytokines, and the produced pro-inflammatory cytokines activate PI3K/AKT and NF-κB signaling *per se* (Ojaniemi et al., 2003; Ozes et al., 1999). However, it is unknown whether PI3K-induced AKT signaling regulates IKK-induced NF-κB signaling and pro-inflammatory cytokine production in the hypothalamus in an LPS-treated animal model. AKT signaling is activated through PI3K-mediated conversion of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], an integral signaling molecule and a minor component of cellular membranes, into phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$]. Cytosolic inactive AKT is recruited to the membrane and engages $PI(3,4,5)P_3$ by interacting with the PH domain. This leads to phosphorylation of T308 and S473 by phosphoinositide-dependent protein kinase 1 and mechanistic target of rapamycin complex 2, respectively, resulting in maximal activation. $PI(4,5)P_2$ is also converted to inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] by phospholipase C (PLC) (Bunney and Katan, 2010). We recently reported that PLC-related catalytically inactive protein (PRIP), which loses the enzymatic activity of PLC, modulates the metabolism of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ and regulates PI3K-mediated AKT signaling (Asano et al., 2017). The PLC enzyme-dead molecule PRIP was originally identified as an Ins$(1,4,5)P_3$-binding protein with domain organization similar to that of PLCδ1 (Kanematsu et al., 1992, 1996, 2000). Although we identified the roles of PRIP in $Ins(1,4,5)P_3$-mediated intracellular Ca^{2+} signaling (Harada et al., 2005; Takeuchi et al., 2000), its involvement in $PI(4,5)P_2$-mediated cell signaling remains unclear. Because PI3K/AKT signaling is correlated with IKK/NF-κB signaling (Bai et al., 2009; Ozes et al., 1999), PRIP may regulate LPS-mediated inflammatory responses and appetite. Therefore, in the present study, we examined the phospholipid signaling-regulated AKT/NF-κB pathway and inflammatory responses in the hypothalamus and elucidated PRIP involvement in the regulation of anorexia using LPS-administered *Prip* knockout mice.

2. Materials and methods

2.1. Animals

Experiments were conducted using 10–15-week-old male mice with similar body weight. Two mammalian homologs of PRIP, PRIP1 and PRIP2, are also known as PLC-like 1 (PLCL1) and PLCL2, respectively (Kanematsu et al., 1996; Uji et al., 2002). *Prip1 (Plcl1)*-KO and *Prip2 (Plcl2)*-KO mouse strains were mated to produce *Prip1* and *Prip2* double knockout (*Prip*-KO) mice and their corresponding wild-type (WT) mice, as described previously (Kanematsu et al., 2002; Mizokami et al., 2007). Briefly, heterozygous ($Prip1^{+/-}$, $Prip2^{+/-}$) mice, both of which were backcrossed to a parental C57BL/6 strain at least 11 times (N11), were mated to generate a *Prip*-KO strain and a corresponding WT strain. Each strain of littermates was mated *inter se*, and *Prip*-KO or WT homozygotes were obtained. To obtain the required number of

experimental mice, we mated each strain of mice *inter se*, and mice beyond the 8 generation were used for the experiments. Mice were reared in a pathogen-free facility at 22 °C–24 °C, with a 12-h light/dark cycle (lights on at 8:00 a.m., lights off at 8:00 p.m.) at Hiroshima University in Japan, and they were fed a normal laboratory diet and water *ad libitum*. This study was approved by the Animal Care and Use Committees of Hiroshima University (permission number: A14-189-1–3), Kyushu University (permission numbers: A19-174 and 26–99), and Daiichi University of Pharmacy (permission numbers: R01-017 and 2019-002) and was performed in accordance with the Guide for Animal Experimentation Regulation of Hiroshima University, Kyushu University, and Daiichi University of Pharmacy.

2.2. LPS administration

An intraperitoneal (ip) injection of LPS (100 μg/kg, 055:B5; Sigma-Aldrich, St. Louis, MO, USA) was at 5 mL/kg. Mice were euthanized via decapitation under anesthesia with pentobarbital (50 mg/kg, ip), and the brain was rapidly dissected out. The hypothalamus was rapidly dissected using mouse brain stereotaxic coordinates; a 3-mm-thick coronal brain slice was obtained with brain matrices (EM Japan, Tokyo, Japan; antero-posterior −3.0 mm to the bregma) and placed in ice-cold PBS; the hypothalamus was then cut in pieces with 3 mm of width (medial-lateral ± 1.5 mm) with 2 mm of thickness from the ventral side. The samples were snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Antibodies

The primary antibodies used were as follows: anti-phospho-STAT3 (Tyr705; #9131, 1:1000), anti-STAT3 (#4904, 1:1000), anti-phospho-AKT (Thr308; #2965, 1:1000), anti-phospho-AKT (Ser473; #4060, 1:1000), anti-AKT (#2920, 1:1000), anti-phospho-IKK α/β (Ser176, Ser180; #2697, 1:1000), anti-IKKα (#2682, 1:1000), anti-IKKβ (#2678, 1:1000), and anti-inhibitor of NF-κB (IκB) (#4814, 1:1000) antibodies were purchased from Cell Signaling Technology, (Danvers, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #MAB374, 1:1000) antibody was purchased from Merck Millipore

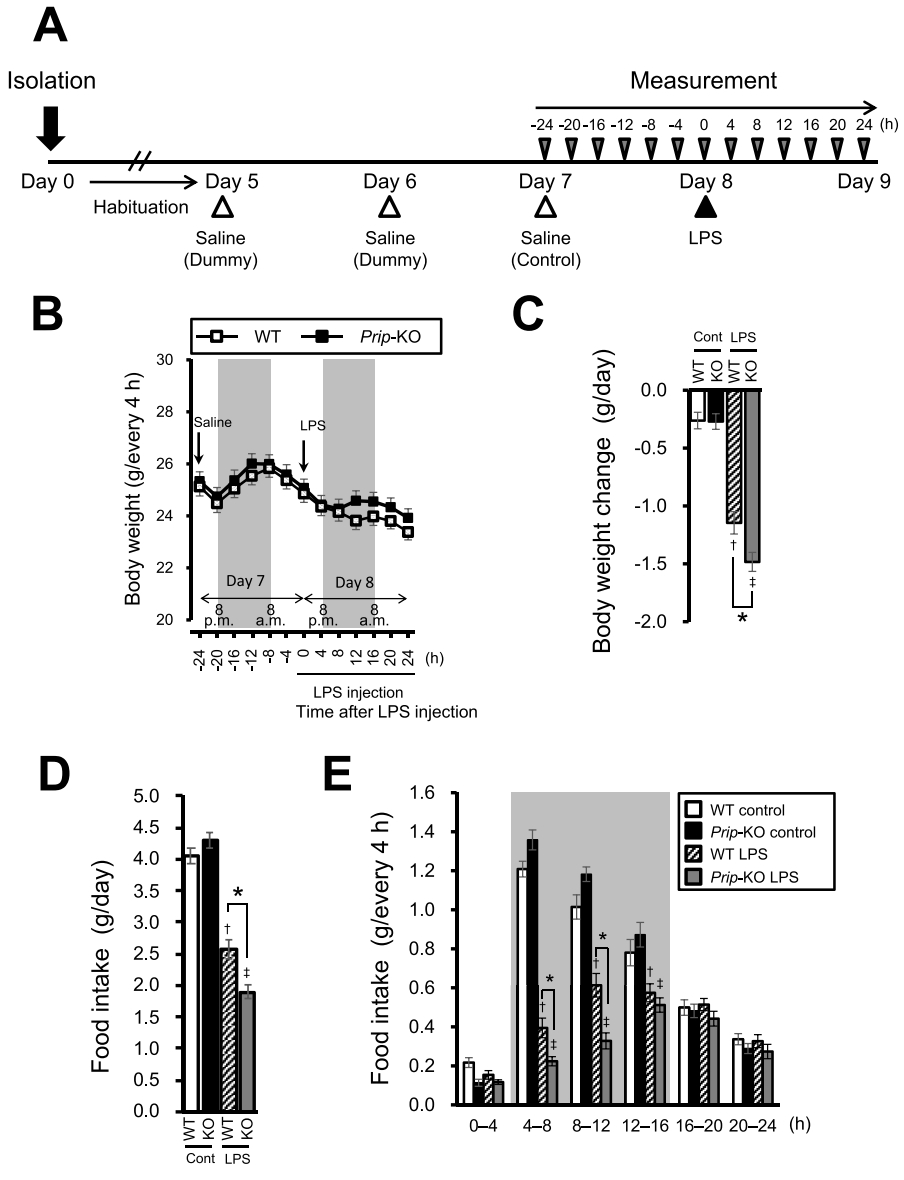

Fig. 1. High-sensitivity phenotype of lipopolysaccharide (LPS)-induced anorexia in *Prip*-KO mice. (A) Schematic timeline representing the experimental design and food intake measurement. Mice were habituated to a novel environment for 5 days followed by an intraperitoneal (ip) saline injection (dummy) twice. The mice were subjected to saline ip injection (control) on day 7 followed by LPS ip injection (100 μg/kg) on day 8. (B and C) Measurement of body weight every 4 h after saline and LPS injection (B) and analysis of body weight change over 24 h (C). (D and E) Cumulative food intake over 24 h (D) and for each 4-h period (E) are shown. Cont, saline-treated mice; LPS, LPS-treated mice; WT, wild-type mice; KO, *Prip*-knockout mice. Gray solid color in B and E indicates the dark phase (from 8:00 p.m. to 8:00 a.m.). Data are presented as the mean ± standard error of the mean (n = 24 for each group). $^{*}p < 0.05$ between the indicated groups, †$p < 0.05$ versus the WT saline value at each time point, ‡$p < 0.05$ versus the KO saline value at each time point (ANOVA with Tukey's honestly significant difference post-hoc comparison).

(Darmstadt, Germany). Anti-PRIP1 and anti-PRIP2 polyclonal antibodies (1:1000) were developed previously (Kanematsu et al., 2002; Mizokami et al., 2007). Horseradish peroxidase-conjugated anti-rabbit IgG (#AP132P, 1:10000) and anti-mouse IgG (#AP124P, 1:10000) secondary antibodies were purchased from Merck Millipore.

2.4. Measurement of food intake

WT and *Prip*-KO mice were housed individually before experiments, with the first day of isolation set as day 0. Saline (5 mL/kg, ip) was injected on days 5 and 6 for habituation to injection. Food intake was manually measured every 4 h, after a saline ip injection at 4:00 p.m. on day 7, followed by an LPS injection (100 μg/kg, ip) at 4:00 p.m. on day 8 (Fig. 1A).

2.5. Preparation of isolated hypothalamic microglia

Microglia were isolated using the MACS system (Miltenyi Biotec, Teterow, Germany) as previously described (Yamawaki et al., 2018). Briefly, the hypothalamus was obtained from WT or *Prip*-KO mice (5 mice per group). Pooled hypothalami were minced in Hank's balanced salt solution (Nacalai Tesque, Kyoto, Japan) and then enzymatically digested using a neural tissue dissociation kit (Miltenyi Biotec) for 35 min at 37 °C. Tissue debris was eliminated with a 70-μm cell strainer, and myelin was eliminated using Myelin Removal Beads II (Miltenyi Biotec). The obtained single cells were magnetically labeled with CD11b micro beads II (Miltenyi Biotec) and loaded onto a MACS column (Miltenyi Biotec), and CD11b-positive cells were isolated as microglia. RNA was extracted from the obtained microglia, using an RNA isolation kit (Arcturus PicoPure RNA Isolation Kit; Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Quantitative real-time polymerase chain reaction (qPCR) analysis

Tissue samples were homogenized in Sepasol RNA I Super G (Nacalai Tesque) at 10,000 rpm with a polytron homogenizer, and total RNA was isolated in accordance with the manufacturer's protocol. cDNA was synthesized from 0.5 μg of total RNA in a final volume of 10 μL, using the ReverTra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo, Osaka, Japan) with a thermal cycler (T Professional Basic Gradient 96; Biometra, Göttingen, Germany). *Gapdh* was considered the internal control. For qPCR analysis, two-step qPCR (Thunderbird SYBR qPCR Mix; Toyobo) was performed with a PikoReal 96-well system (Thermo Fisher Scientific). The cycling protocol was as follows: DNA polymerase activation at 95 °C for 1 min, followed by denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min, for 40 cycles. Gene expression was normalized to that of *Gapdh* mRNA in the same samples, using the $2^{-\Delta\Delta Ct}$ method. qPCR was performed using the following primers: *Gapdh*, forward 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse 5′-GTAGACCATGTAGTTGAGGTCA-3′; *Il-6*, forward 5′-ACAAC CACGGCCTTCCCTACTT-3′, reverse 5′-CACGATTTCCCAGAGAACAT GTG-3′; *Il-1b*, forward 5′- AACCTGCTGGTGTGTGACGTTC-3′, reverse 5′-CAGCACGAGGCTTTTTTGTTGT-3′; *Tnf-a*, forward 5′-GGGGCCACCA CGCTCTTCTGTC-3′, reverse 5′- TGGGCTACAGGCTTGTCACTCG-3′; *Suppressor of cytokine signaling 3* (*Socs3*), forward 5′-GAGATTTCGCTT CGGGACTA-3′, reverse 5′-GCTGGTACTCGCTTTTGGAG-3′; cyclooxygenase-2 (*Cox2)*, forward 5′-CCACTTCAAGGGAGTCTGGA-3′, reverse 5′-AGTCATCTGCTACGGGAGGA-3′, ionized calcium-binding adapter molecule 1 (*Iba1*), forward 5′-TGGTCCCCCAGCCAAGA-3′, reverse 5′-CCCACCGTGTGACATCCA-3′; *Prip1*, 5′-TGAGAATGGGGAAGAAA GTT-3′, reverse 5′-TCTATGGCTTCTCGTAAGGG-3′; *Prip2*, 5′-ACTGTG GCTATGTTCTTCGA-3′, reverse 5′-TTTGATGTGAAGCAACTGAG-3′.

2.7. Reverse-transcription PCR (RT-PCR) analysis

RT-PCR analysis was performed using a PCR master mix (QuickTaq HS Dye Master Mix; Toyobo) with a thermal cycler (T Professional Basic Grafient96; Biometra). The primer sequences were the same as those used for qPCR. The cycling protocol was as follows: DNA polymerase activation at 94 °C for 2 min, followed by denaturation at 94 °C for 30 s, and annealing/extension at 55 °C for 30 s, for 35 cycles (*Prip1, Prip2,* and *Iba1*) or 20 cycles (*Gapdh*). After PCR, the products were mixed with an intercalator (UltraPower DNA Safedye; Gellex International, Tokyo, Japan) and separated on a 2% agarose gel in 1 × Tris-acetate-EDTA buffer. Amplified products were captured with a gel imaging system (Atto Corporation, Tokyo, Japan).

2.8. Western blotting

Lysates were prepared using a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 0.1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (#25955, Nacalai Tesque) and a phosphatase inhibitor cocktail (#07574, Nacalai Tesque). The samples were centrifuged at 20,600 × *g* for 30 min at 4 °C, and the supernatants were harvested. Protein concentration was determined with a protein assay bicinchoninate kit (#06385, Nacalai Tesque). The samples were fractionated using SDS-PAGE and electro-transferred onto a polyvinylidene fluoride membrane. Membranes were subsequently blocked with 5% skimmed milk for 3 h (for phospho-STAT3 detection) or for 1 h (other antibodies), followed by incubation with each primary antibody overnight at 4 °C. Antibodies were diluted with 2% skimmed milk. The membranes were washed with Tris-buffered saline with Tween 20 and incubated with a respective horseradish peroxidase-conjugated secondary antibody for primary antibody recognition. Immunoreactivity was detected with an electrochemiluminescence reagent (ECL; Promega, Fitchburg, WI, USA) using the ImageQuant LAS 4000 mini imager (GE Healthcare, Chicago, IL, USA). The density of immunoreactive bands was measured using Image J 1.50v software (Wayne Rasband; NIH, Bethesda, MD, USA).

2.9. Enzyme-linked immunosorbent assay (ELISA) for plasma cytokine quantification

Up to 150 μL blood was sampled from the orbital plexus of WT and *Prip*-KO mice under isoflurane anesthesia before and 1 h after LPS (100 μg/kg, ip) administration. Plasma prepared from the blood samples with EDTA was assayed for IL-1β (#KE10003, Proteintech, Rosemont, IL, USA), IL-6 (#431307, Biolegend, San Diego, CA, USA), and TNF-α (#430907, Biolegend) with ELISA kits in accordance with the manufacturer's instructions.

2.10. Statistical analysis

JMP 8.0.2 (SAS, Cary, NC, USA) was used for statistical analyses. Data are expressed as mean ± standard error of the mean values. Student's *t*-test or ANOVA with Tukey's honestly significant difference post-hoc comparison was used. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Prip-KO mice showed a high-sensitivity phenotype to LPS-induced anorexia

To investigate whether PRIP deficiency in mice affects LPS-induced anorexia, we measured *ad libitum* food intake every 4 h for 24 h after saline ip injection (control) followed by the measuring of food intake every 4 h after LPS (100 μg/kg, ip) injection (Fig. 1A). Body weight was measured during the food intake measurement (Fig. 1B). Mean weight change was similar between *Prip*-KO and WT mice after the saline injection on day 7; however, significant weight decrease was observed in the two genotypes after LPS administration on day 8 compared with the

respective saline controls on day 7 (Fig. 1C). Notably, greater weight loss was observed in *Prip*-KO mice than in WT mice ($p = 0.0192$). The saline-administered WT and *Prip*-KO mice showed similar cumulative 24-h food intake (Fig. 1D) and food intake for each 4-h period (Fig. 1E). In contrast, after the subsequent LPS injection, the total 24-h food intake decreased significantly by approximately 65% and 46% in WT and *Prip*-KO mice, respectively, compared with food intake in the respective saline controls (day 7). Importantly, food intake reduction in *Prip*-KO mice was significantly higher than that in WT mice (Fig. 1D; $p = 0.0019$). Moreover, differences were observed in the food intake on day 8 during the 4–8 h and 8–12 h periods after LPS injection ($p = 0.029$ and $p < 0.001$, respectively; Fig. 1E), suggesting that *Prip*-KO mice are susceptible to LPS-mediated inflammatory responses.

3.2. Higher gene expression of pro-inflammatory cytokines in Prip-KO hypothalamus

LPS-induced anorexia is implicated in the elevation of hypothalamic pro-inflammatory cytokines (Jang et al., 2010; Ogimoto et al., 2006; Wisse et al., 2007). Therefore, we analyzed the gene expression of IL-1β, IL-6, and TNF-α in the hypothalamus by qPCR. The expression of *Il-1b*, *Il-6,* and *Tnf-a* was upregulated in the both genotypes at 2 h after peripheral LPS injection. However, the gene expression in *Prip*-KO mice was significantly higher than that in WT mice at 2 h (*Il-1b, Il-6, and Tnf-a*; $p = 0.0016$, $p < 0.0001$, and $p < 0.0001$, respectively) after LPS administration (Fig. 2). These results suggest that over-expression of pro-inflammatory cytokines occurred in the hypothalamus in an early phase (~2 h) of peripherally administered LPS-induced neuroinflammation, which causes a severe anorectic phenotype in *Prip*-KO mice.

3.3. Similar plasma levels of peripheral inflammatory biomarkers in WT and Prip-KO mice

Peripheral inflammation induces brain dysfunction (Dantzer et al., 2008); therefore, differences in the degree of peripheral inflammatory responses may influence brain inflammation. Pro-inflammatory cytokine mRNAs were upregulated 2 h after LPS ip injection in the hypothalamus in *Prip*-KO rather than WT mice (Fig. 2). Therefore, we quantified cytokine levels (IL-1β, IL-6, and TNF-α) in peripheral blood via ELISA, 1 h after LPS (100 μg/kg, ip) injection. The plasma levels of IL-1β and IL-6 were slightly but not significantly increased in WT rather than *Prip*-KO mice; in contrast, that of TNF-α was slightly but not significantly increased in *Prip*-KO rather than WT mice (Table 1). These data suggest that peripheral inflammation in WT and *Prip*-KO mice similarly influences the CNS.

3.4. PRIP deficiency enhanced the NF-κB pathway and AKT phosphorylation in the hypothalamus

NF-κB is required for induction of a several inflammatory genes, including *Il-1b*, *Il-6,* and *Tnf-a* (Liu et al., 2017). The LPS-induced production of these pro-inflammatory cytokines in the hypothalamus causes anorexia (Jang et al., 2010). IκB is phosphorylated by IKK, which induces the subsequent ubiquitination and degradation of IκB, followed by promotion of NF-κB activation (Karin and Ben-Neriah, 2000). IKK is activated by AKT as well as NF-κB-inducing kinase (NIK) and transforming growth factor β-activated kinase in an immune challenge (Akira and Takeda, 2004; Ozes et al., 1999). Thus, the PI3K/AKT pathway may affect LPS-induced inflammatory responses in the CNS. To investigate the involvement of NF-κB signaling in PRIP-regulated PI3K/AKT signaling, we analyzed IKKα/β phosphorylation and IκB degradation in the hypothalamus obtained from LPS-treated mice. IKKα/β phosphorylation in *Prip*-KO mice was significantly higher than that in WT mice (Fig. 3A and B; $p = 0.0056$ versus total IKKα and $p = 0.0016$ versus total IKKβ). Consistently, IκB level was significantly downregulated (Fig. 3A and C; $p = 0.0033$). The phosphorylation of AKT at T308 and S473, which is required for full activation of AKT (Manning and Toker, 2017), increased significantly in the *Prip*-KO hypothalamus, compared with that in the WT hypothalamus (Fig. 3A and D; $p = 0.0129$ and $p = 0.0038$, respectively). These findings indicate that PRIP deficiency enhances the activation of AKT-mediated NF-κB signaling in the hypothalamus.

Table 1
Plasma cytokine levels in wild-type and *Prip*-knockout mice.

	Genotype	Control	LPS	WT vs KO in LPS
IL-1β	WT	6.8 ± 2.9	43.0 ± 12.4*	n.s.
(pg/mL)	KO	5.7 ± 2.9	20.7 ± 3.9	
IL-6	WT	0.03 ± 0.03	15.8 ± 4.3*	n.s.
(ng/mL)	KO	0.12 ± 0.06	10.7 ± 0.9*	
TNF-α	WT	1.92 ± 0.8	1116.9 ± 250.2*	n.s.
(pg/mL)	KO	2.59 ± 0.4	1537.8 ± 207.5*	

Plasma cytokine levels in wild-type (WT) and *Prip*-KO (KO) mice were quantified before (control) and 1 h after intraperitoneal injection of 100 μg/kg of lipopolysaccharide (LPS). Data are presented as mean ± standard error of the mean values (n = 5 for each group). *$p < 0.05$, the LPS group vs the corresponding control group; n.s. (not significant), WT value vs KO value in LPS treatment (ANOVA with Tukey's honestly significant difference post-hoc comparison).

3.5. PRIP deficiency upregulates STAT3 phosphorylation and Socs3 and Cox2 gene expression in the hypothalamus

Hypothalamic STAT3 phosphorylation is associated with hypophagia, and peripheral LPS administration promotes pro-inflammatory cytokine expression followed by STAT3 phosphorylation in the hypothalamus with a reduction in food intake (Vaisse et al., 1996; Yamawaki et al., 2010). STAT3 is activated by many cytokines, including IL-6, IL-1β, and TNF-α, and a major IL-6-driven signaling pathway involves JAK-dependent STAT3 activation (Aggarwal et al., 2009). We examined STAT3 phosphorylation using LPS-administered

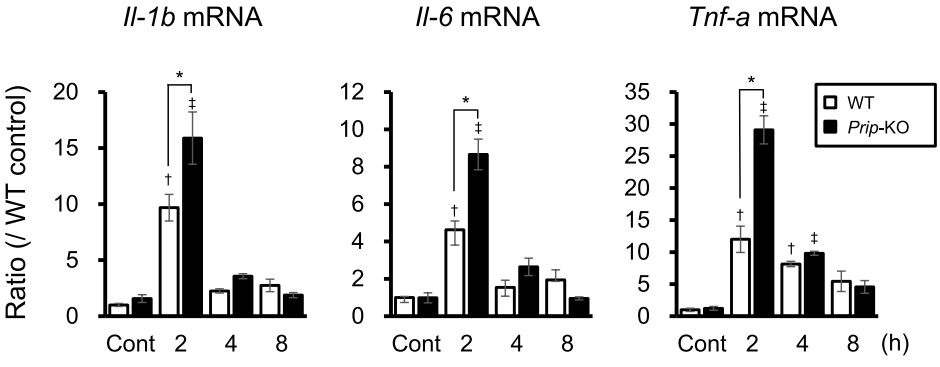

Fig. 2. The gene expression of pro-inflammatory cytokines is upregulated in *Prip*-KO hypothalamus after peripheral lipopolysaccharide (LPS) injection. Time-dependent changes in the expression of *Il-1b*, *Il-6,* and *Tnf-a* in the hypothalamus of wild-type (WT) and *Prip*-KO mice after intraperitoneal LPS injection (100 μg/kg). Gene expression was evaluated with qPCR methods. Data are presented as the mean ± standard error of the mean (n = 5). *$p < 0.05$ between the indicated groups, †$p < 0.05$ versus the WT control value at each time point, ‡$p < 0.05$ versus the *Prip*-KO control value at each time point (ANOVA with Tukey's honestly significant difference post-hoc comparison).

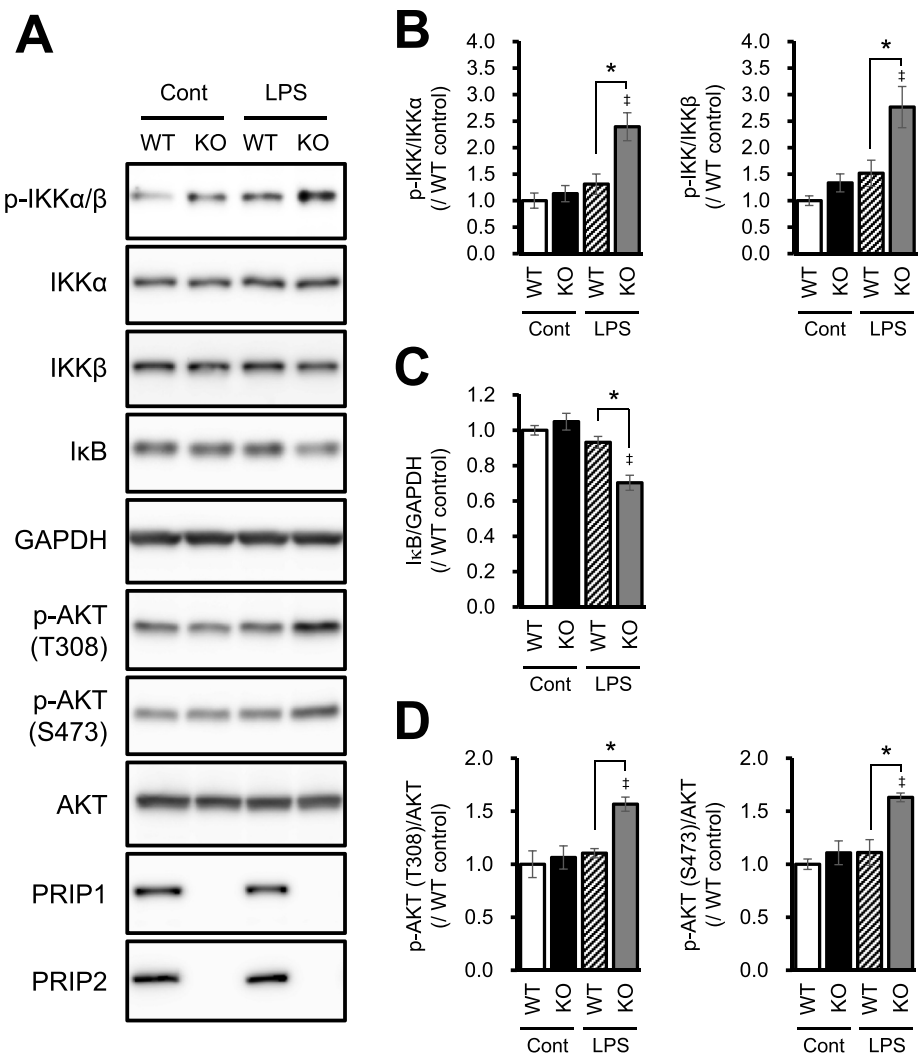

Fig. 3. PRIP deficiency upregulates NF-κB signaling mediated by AKT-IKK signaling. Hypothalamus samples were obtained 2 h after the intraperitoneal injection of saline (cont) or LPS (100 μg/kg). (A) Western blotting was performed using indicated antibodies. Representative blot images are shown. GAPDH was used as a loading control. (B–D) Quantitation of combined phosphorylation of IKKα and IKKβ was analyzed against total IKKα and IKKβ levels detected using the corresponding pan-antibodies, respectively, and expressed as p-IKK/IKKα and p-IKK/IKKβ (B). Quantitation of immunodensity in the degradation of IκB and phosphorylation levels of AKT (T308 and S473) was analyzed against GAPDH (C) and total AKT (D), respectively. Data are presented as the mean ± standard error of the mean (n = 5 for each group). *$p < 0.05$ between the indicated groups. ‡$p < 0.05$ versus the *Prip*-KO control value (ANOVA with Tukey's honestly significant difference post-hoc comparison).

Prip-KO and WT hypothalamus. STAT3 phosphorylation was significantly increased in both genotypes 2 h–4 h (WT mice) or 2 h–8 h (*Prip*-KO mice) after peripheral LPS injection (Fig. 4A and B). The increase in phosphorylation in *Prip*-KO mice was significantly higher than that in WT mice at 2 h, 4 h, and 8 h (Fig. 4B; $p = 0.0015$, $p < 0.001$, and $p = 0.023$, respectively). LPS administration did not alter PRIP1 and PRIP2 expression levels in the hypothalamus (Fig. 4C). However, the hypothalamic STAT3 activation upregulated the gene expression of the downstream signaling molecule SOCS3, a negative feedback regulator for STAT3 signaling, in *Prip*-KO mice (2–8 h after LPS administration) and WT mice (2–4 h after LPS administration) (Fig. 4D). Importantly, the upregulation in *Prip*-KO hypothalamus was greater at 4 h ($p = 0.0396$) and 8 h ($p = 0.0830$) after LPS administration than those in WT mice.

The expression of *Cox2*, an important player in LPS-mediated anorexia (Lugarini et al., 2002), is regulated by NF-κB and STAT3 signaling (D'Acquisto et al., 1997; Rummel et al., 2006). Therefore, to examine the downstream activation of NF-κB and STAT3 signaling, we next investigated hypothalamic *Cox2* expression. The expression of *Cox2* was significantly increased during 2–8 h after peripheral LPS injection in the two phenotypes. *Cox2* expression in *Prip*-KO mice was significantly higher than that in WT mice at 2 h and 8 h (Fig. 4E; $p = 0.0057$ and $p = 0.0002$ at 4 h and 8 h, respectively) or substantially high at 4 h, suggesting that the neuroinflammation response is more severe in *Prip*-KO hypothalamus than in WT mice. These data indicate that a severe anorexia phenotype in *Prip*-KO mice depends on NF-κB and STAT3 acceleration-dependent signaling in the hypothalamus.

3.6. High expression of pro-inflammatory cytokines in Prip-KO hypothalamic microglia

The hypothalamus is composed of many different cell types, including microglia, and activated microglia are involved in aggravating neuroinflammation. To investigate microglial contribution in the accelerated inflammatory responses in *Prip*-KO hypothalamus, we analyzed the expression of *Iba1*, a marker of active microglia. *Iba1* expression increased 24 h after LPS administration, and the elevation was more prominent in *Prip*-KO hypothalamus than that in WT mice (Fig. 5A), suggesting that peripheral LPS application intensely activates *Prip*-deficient microglia. We next examined pro-inflammatory cytokine expression in hypothalamic microglia isolated from LPS-treated mice using the MACS system. The isolated microglia expressed *Prip1* and *Prip2* genes (Fig. 5B). Pro-inflammatory cytokine expression was higher in *Prip*-KO hypothalami than those in WT mice (Fig. 5C; *Il-1b*, *Il-6*, and *Tnf-α*; $p = 0.0078$, $p = 0.0096$, and $p = 0.0004$, respectively).

4. Discussion

Anorexia is a hallmark of systemic inflammation, and inflammation-associated anorexia is primarily a result of cytokine action in the CNS. It is known that peripheral injection of LPS induces anorexia with elevation of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α

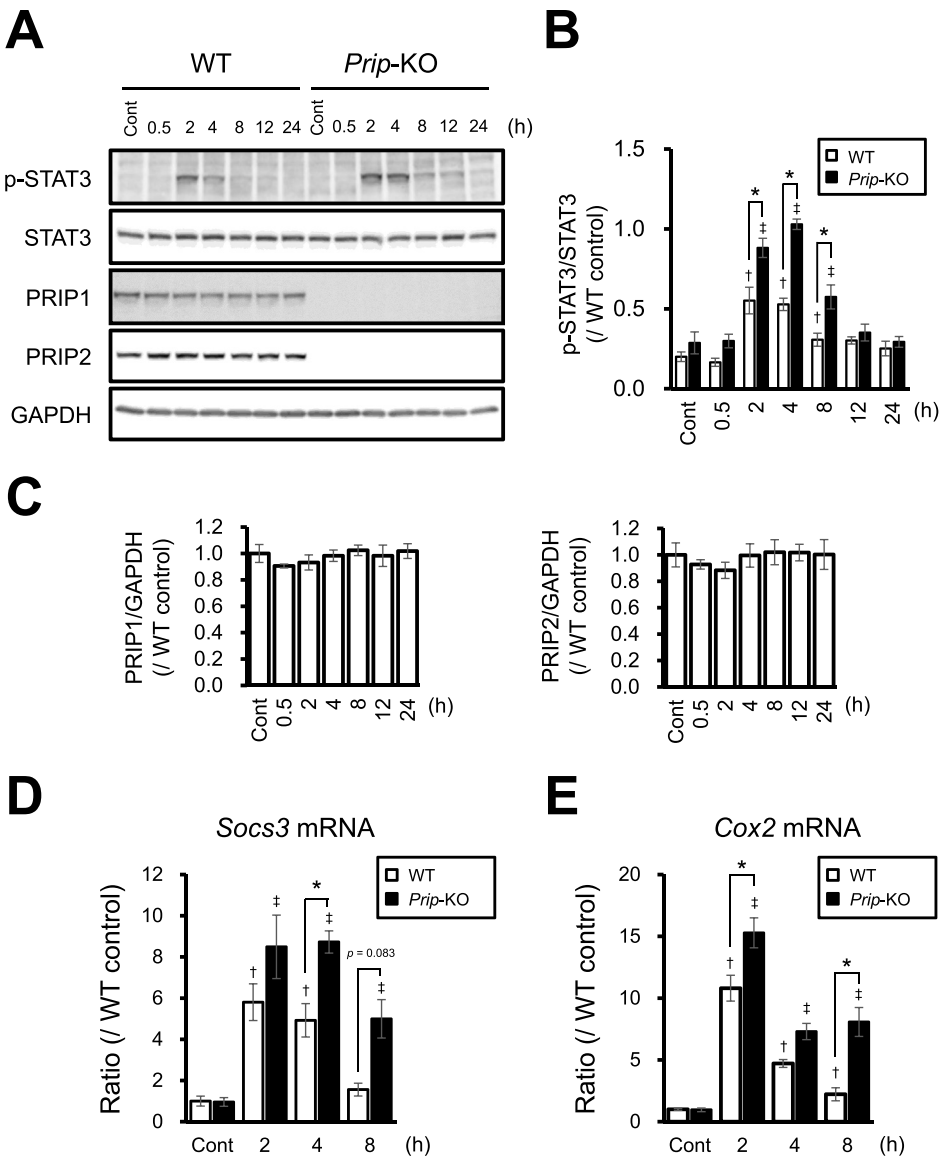

Fig. 4. *Prip* deficiency upregulates STAT3 signaling in the hypothalamus. (A–C) Time-dependent changes in the phosphorylation levels of STAT3 (A and B) and the expression of PRIP1 and PRIP2 (A and C) in the hypothalamus of wild-type (WT) and *Prip*-KO mice after intraperitoneal (ip) lipopolysaccharide (LPS) injection (100 μg/kg). Western blotting was performed using the indicated antibodies. Representative blot images are shown (A). Results of quantitative immunodensity expressed as changes in phospho-STAT3 against total STAT3 levels (B) and of PRIP1 and PRIP2 levels in WT hypothalamus against the level of corresponding GAPDH, a loading control (C). Data are presented as the mean ± standard error of the mean (n = 5). (D and E) Results of qPCR analyses for *Socs3 and Cox2* expression in the hypothalamus. Data are presented as the mean ± standard error of the mean (n = 5 for each group). *$p < 0.05$ between the indicated groups, †$p < 0.05$ versus the WT control value at each time point, ‡$p < 0.05$ versus the *Prip*-KO control value at each time point (ANOVA with Tukey's honestly significant difference post-hoc comparison).

in peripheral and CNS. In addition, the peripheral or intracerebroventricular injections of pro-inflammatory cytokines IL-1β and TNF-α cause anorexia in rodents (Elander et al., 2007; Fantino and Wieteska, 1993; Harden et al., 2008; Michie et al., 1989). Therefore, circulating cytokines in the body and *de novo* production of cytokines in the CNS are widely viewed as mediators of inflammatory anorexia. Inflammatory responses through PI3K/AKT signaling, which are activated by LPS, IL-1β, and TNF-α, may promote NF-κB activation and anorexia in an animal model (Bai et al., 2009; Ojaniemi et al., 2003; Ozes et al., 1999). In this study, we examined whether PRIP, a new molecule negatively regulating phosphatidylinositol metabolism-dependent AKT signaling, is involved in inflammation-associated anorexia, and elucidated that deficiency in PRIP increases pro-inflammatory cytokine expression in hypothalamic microglia and promotes LPS-mediated anorectic response in mice.

Pro-inflammatory cytokines in peripheral blood are potent mediators that link the periphery and CNS in LPS ip injection-elicited brain inflammation and anorexia; consequently, upregulation of hypothalamic pro-inflammatory cytokines causes LPS-induced anorexia. In our experiments, levels of circulating pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) did not differ between WT and *Prip*-KO mice 1 h after LPS injection. However, mRNA levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) were significantly higher in the hypothalamus in *Prip*-KO rather than WT mice 2 h after LPS injection. These data suggest that local hypothalamic inflammatory responses vary in *Prip*-KO mice. Therefore, we investigated the role of PRIP in anorexia caused by LPS-induced inflammation in the hypothalamus, a key brain region regulating food intake.

Inflammatory stimuli, such as LPS and pro-inflammatory cytokines, induce inflammatory responses through PI3K-activated AKT signaling, which regulates NF-κB activation (Ozes et al., 1999; Ojaniemi et al., 2003). AKT phosphorylates and activates two subtypes of IKKα and IKKβ, leading to IκB degradation and NF-κB activation (Bai et al., 2009; Ouyang et al., 2006; Vandermoere et al., 2005). The transcription factor NF-κB is activated downstream of LPS and pro-inflammatory cytokines (Taniguchi and Karin, 2018) which results in inflammatory response and anorexia after LPS administration (Jang et al., 2010). NF-κB, whose activation is regulated by the IKK-mediated degradation of IκB, promotes the transcriptional activity of various pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6 (Libermann and Baltimore, 1990; Luo and Zheng, 2016). We observed that IKK phosphorylation-induced IκB degradation increased in *Prip*-KO hypothalamus and enhanced AKT signaling activation, indicating that the upregulation of pro-inflammatory cytokines in the *Prip*-KO hypothalamus resulted from

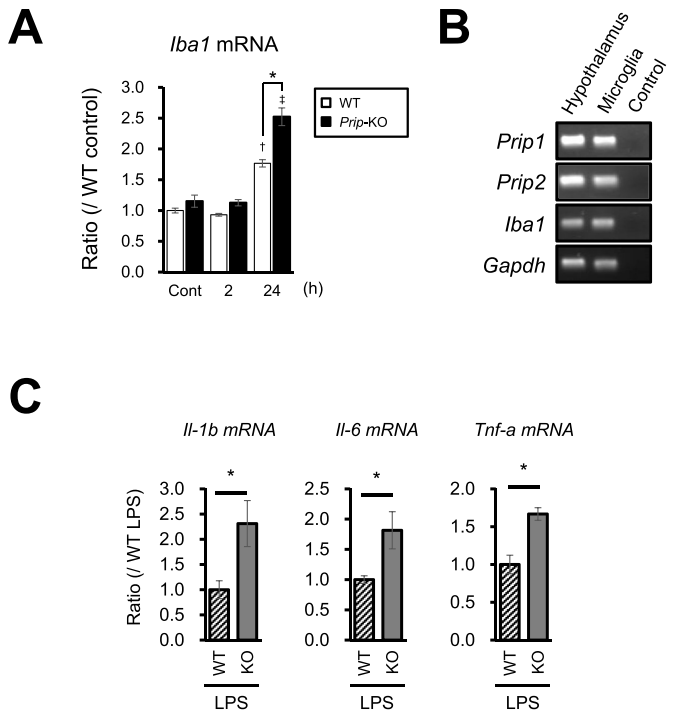

Fig. 5. Gene expression of pro-inflammatory cytokines is upregulated in *Prip*-KO hypothalamic microglia after peripheral lipopolysaccharide injection. (A) Gene expression of *Iba1* in WT and *Prip*-KO hypothalami 2 h and 24 h after saline (cont) or lipopolysaccharide (LPS) injections. (B) Expression of *Prip1*, *Prip2*, and *Iba1* genes in isolated hypothalamic microglia (Microglia) was examined by reverse-transcription polymerase chain reaction. Hypothalamus was used as a positive control. *Iba1* and *Gapdh* were used as a microglial marker and an internal control, respectively. Control lane (no template) indicates a negative control. (C) Pro-inflammatory cytokine expression was evaluated with qPCR methods using isolated hypothalamic microglia from WT and *Prip*-KO mice 2 h after LPS injection. Data are presented as the mean ± standard error of the mean (n = 5). $*p < 0.05$ between the indicated groups (Student's *t*-test).

increased NF-κB signaling. *Prip*-KO mice exhibited higher expression of pro-inflammatory cytokines in the hypothalamus 2 h after LPS peripheral injection compared with that in WT mice, suggesting that regional inflammation responses in *Prip*-KO hypothalamus were upregulated. Therefore, we conclude that the deficiency of PRIP, a negative regulator of AKT signaling via PI3K-mediated PI(3,4,5)P_3 production (Asano et al., 2017), shows increased LPS-induced AKT and IKKα/β phosphorylation followed by increased NF-κB-regulated inflammatory responses, thus causing a worse anorexia phenotype than that of WT mice.

Although pro-inflammatory cytokine expression in WT hypothalamus increased 2 h after peripheral LPS administration (Fig. 2), a distinct activation of IKK-IκB degradation-mediated NF-κB signaling was not observed between the groups of saline-administration (control) and LPS-administration in WT mice (Fig. 3). It is reported that LPS ip injection (200 μg/kg) induces anorexia via activation of IKK-NF-κB signaling in the WT hypothalamus (Jang et al., 2010). However, we used a dose of 100 μg/kg LPS (ip) to induce peripheral inflammation in this study, which may be too low to detect a change in IKK-NF-κB signaling in western blotting analysis.

In addition to NF-κB activation, hypothalamic STAT3 activation is involved in appetite. Peripheral LPS injection increases the expression of IL-6 as well as IL-1β and TNF-α in the hypothalamus (Jang et al., 2010) and induces hypothalamic STAT3 activation (Hosoi et al., 2004). The central inhibition of NF-κB signaling prevents LPS-induced anorexia (Jang et al., 2010), whereas the central inhibition of the JAK-STAT pathway fails to prevent anorexia (Damm et al., 2013). Furthermore, the central inhibition of NF-κB decreases IL-6 expression in the hypothalamus (Jang et al., 2010), and intraventricular injection of IL-6 accelerates IL-1β injection-mediated inhibition of food intake in rats (Harden et al., 2008). IL-6 activates gp130/JAK pathway and phosphorylates STAT3 in the hypothalamus, thereby regulating food intake (Takeda et al., 1999; Vaisse et al., 1996). In contrast, deficiency of IL-1β, IL-6, or TNF-α does not protect against LPS-induced anorexia (Arsenijevic et al., 2000; Fantuzzi et al., 1996; Fattori et al., 1994), although MyD88 deficiency completely abolishes LPS-induced anorexia and prevents hypothalamic pro-inflammatory cytokine elevation (Ogimoto et al., 2006; Yamawaki et al., 2010). These data suggest that LPS-activated NF-κB signaling is essential for an anorectic effect through the production of pro-inflammatory cytokines, and IL-6-activated STAT3 signaling is needed to exacerbate these anorectic effects. Thus, pro-inflammatory cytokine production is involved in LPS-induced anorectic responses. In our study, STAT3 phosphorylation in the hypothalamus of *Prip*-KO mice was higher than that in WT mice 4–8 h after LPS ip injection. The expression of pro-inflammatory cytokines at 2 h was markedly increased in *Prip*-KO hypothalamus compared with that in WT mice, and augmented hypophagia was observed during 4–12 h. Furthermore, the expression of *Socs3*, a negative feedback regulator for STAT3 signaling that is positively regulated by STAT3 activation (Carow and Rottenberg, 2014), was increased in *Prip*-KO hypothalamus 4–8 h after LPS ip injection. These time-course studies indicate that STAT3 phosphorylation regulates the duration of inflammation-induced anorectic effects. Taken together, the LPS-inducible appetite-suppressive phenotype in *Prip*-KO mice may be attributed to alterations in AKT signaling, which regulates NF-κB-mediated pro-inflammatory cytokine expression signaling, followed by STAT3 signaling-mediated anorectic responses.

Microglia, the brain-resident immune cells, are emerging as central players in the regulation of CNS inflammation. We show that isolated hypothalamic microglia expressed more pro-inflammatory cytokines 2 h after peripheral LPS injection in *Prip*-KO mice than those in WT mice, although the higher expression of *Iba1*, a marker of activated microglia, was observed in *Prip*-KO hypothalamus 24 h after peripheral LPS injection. Because *Iba1* expression in the brain is delayed despite microglial activation in response to peripheral LPS injection (Yamawaki et al., 2018), *Prip*-KO microglia may have a higher ability for producing pro-inflammatory cytokines than WT microglia 2 h after LPS injection because of their abnormal AKT signaling. However, further studies are required to investigate the *Prip*-KO microglia-induced inflammation in LPS-induced anorexia. Together, the present results indicate that *Prip* deficiency enhances pro-inflammatory cytokine-induced brain inflammation by activating the AKT/NF-κB pathway in hypothalamic microglia. This, in turn, increases pro-inflammatory cytokine-mediated STAT3 phosphorylation. This sequential signal activation induces the up-regulation of molecules regulating anorexia, such as COX2 in the hypothalamus, resulting in a severe anorexia phenotype in *Prip*-KO mice.

PRIP has several binding partners other than Ins(1,4,5)P_3 and PI(4,5)P_2; these are $GABA_A$ receptor associated protein (Kanematsu et al., 2002), $GABA_A$ receptor β subunit (Terunuma et al., 2004), phosphorylated AKT (Fujii et al., 2010), and PP1 and PP2A (Kanematsu et al., 2006; Yoshimura et al., 2001). PRIP exerts its physiological functions by binding to these proteins and modulating their functions. *Prip*-KO mice exhibited an anti-obesity phenotype in spite of having a higher food intake than WT mice. We determined that this phenotype results from a higher energy expenditure rate in *Prip*-KO mice than in WT mice (Okumura et al., 2014; Oue et al., 2016, 2017). Energy expenditure in non-shivering thermogenesis is controlled by PRIP-dependent recruitment of protein phosphatase activity to the lipid droplet membrane in brown adipocytes. Thus, we do not believe that LPS-induced acute anorectic responses in *Prip*-KO mice are derived from the PRIP-regulating peripheral non-shivering thermogenic pathway. However, PRIP binds to phosphorylated AKT and regulates the intracellular trafficking of $GABA_A$ receptor-containing secretary vesicles (Fujii et al.,

2010). Therefore, a PRIP/phospho-AKT complex potentially modulates the AKT/IKK/NF-κB signaling pathway. Further studies are required to clarify the role of PRIP in the interrelation between AKT and NF-κB signaling pathways in the hypothalamus.

This study shows that PRIP represses the neuroinflammatory response via constitutive inhibition of PI3K/AKT signaling in the brain. Our findings implicate PRIP as a novel regulator of inflammatory brain responses. Currently, no interventions are available to completely control the neuroinflammation that leads to brain dysfunction in patients with infectious diseases. Our findings provide evidence indicating that a unique molecule, PRIP, regulates neuroinflammation. A better understanding of this process might aid the development of therapeutics against infection-induced inflammation in the brain.

Funding

This work was supported by JSPS KAKENHI (grant numbers 15K19731 and 17K11670 to Y.Y., 17H01595 and 17K19766 to M.H., 16K11503 to T.K.) and the Strategic Research Program for Brain Sciences from Japan Agency for Medical Research and Development (AMED; grant number 16dm0107093h0001 to S.Y.).

Author contributions

Y.Y. designed the study, performed the experiments, and drafted the manuscript. S.S., M.A., K.N., H.I., and H.A. performed some experiments. S.A. and K.O. contributed reagents and analytical tools. S.Y. and M.H. helped conduct the study. T.K. conceived and coordinated the study and wrote the manuscript. All authors have read and approved the final manuscript.

Declaration of competing interest

None.

Acknowledgments

We wish to acknowledge the Institute of Laboratory Animal Science (Hiroshima University) for their support in conducting all the animal experiments involved in the current study.

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