Deficiency of PTP1B Attenuates Hypothalamic Inflammation via Activation of the JAK2-STAT3 Pathway in Microglia

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1. Introduction

Obesity is a typical lifestyle-related disease (Stein and Colditz, 2004). The disrupted balance between energy intake and energy expenditure causes obesity, which is defined as the excess accumulation of fat mass (Friedman, 2009). Diet-induced obesity is associated with inflammation not only in the peripheral tissues but also in the hypothalamus (De Souza et al., 2005), by which energy balance is primarily regulated (Morton et al., 2006). The consumption of a high-fat diet (HFD) increases the expression levels of tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) in the arcuate nucleus and lateral hypothalamus (Thaler et al., 2012; De Souza et al., 2005), leading to leptin resistance (De Souza et al., 2005; Zhang et al., 2008; De Git and Adan, 2015). The hypothalamic inflammation induced by a HFD is reported to occur prior to substantial body weight gain, suggesting that it might play a causal role in obesity (Valdearcos et al., 2014; Thaler et al., 2012; De Git and Adan, 2015). Recent studies suggest that glial cells, including microglia, are involved in the inflammatory processes in response to a HFD (De Git and Adan, 2015; Argente-Arizon et al., 2015), although the precise mechanisms by which glial cells regulate hypothalamic inflammation remain to be elucidated.

Protein tyrosine phosphatase 1B (PTP1B) is a non-receptor tyrosine phosphatase that is widely expressed in the body, and negatively regulates leptin signaling by dephosphorylating Janus Activating Kinase 2 (JAK2) in the hypothalamus (Tsou and Bence, 2012; Myers et al., 2001; Zhang et al., 2015). Animals under HFD conditions show increased levels of JAK2 phosphorylation (p-JAK2) in the hypothalamus (Tsou and Bence, 2012; Myers et al., 2001; Zhang et al., 2015). The hypothalamic inflammation induced by a HFD is reported to occur prior to substantial body weight gain, suggesting that it might play a causal role in obesity (Valdearcos et al., 2014; Thaler et al., 2012; De Git and Adan, 2015). Recent studies suggest that glial cells, including microglia, are involved in the inflammatory processes in response to a HFD (De Git and Adan, 2015; Argente-Arizon et al., 2015), although the precise mechanisms by which glial cells regulate hypothalamic inflammation remain to be elucidated.

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macrophages, myeloid cells and microglia (Zhang et al., 2013; Grant et al., 2014; Pike et al., 2014; Song et al., 2016). However, the role of PTP1B in hypothalamic inflammation induced by a HFD has yet to be clarified.

In the present study, we employed PTP1B KO mice and investigated the role of PTP1B in hypothalamic inflammation induced by a HFD.

2. Materials and Methods

2.1. Animals

PTP1B−/− mice (Klanman et al., 2000; Zabolotny et al., 2002) were produced by intercrossing male and female heterozygotes; their PTP1B+/− littermates were used as control mice. All animal procedures were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine and performed in accordance with the institutional guidelines that conform to the National Institutes of Health animal care guidelines. Mice were maintained on a 12 h light/12 h dark cycle in a temperature-controlled barrier facility, with free access to water and food. Age-matched littermates were used for all experiments.

2.2. Food and body composition

At weaning (3 weeks old), male mice were placed on diets of either a standard chow (CE-2, CLEA Japan, Tokyo, Japan; 24.9% protein, 4.6% fat and 70.5% carbohydrate) or a custom high fat diet (Test Diet 58Y1, PMI Nutrition International, KS, USA; 18.3% protein, 60.9% fat, and 20.1% carbohydrate). The composition of fats in the high fat diet was as follows: 39.2% total saturated fatty acids, 40.1% total monounsaturated fatty acids, 13.5% linoleic acid, 1.1% linolenic acid, 0.2% arachidonic acid and 1.1% omega-3 fatty acids. Body weight was monitored until the age of 16 weeks.

2.3. Measurement of Epididymal Fat Pad Weight and Serum TNFα Levels

Measurement of epididymal fat pad weight and collection of blood for measuring serum TNFα levels from mice were performed at the age of 7 weeks in the beginning of the light cycle (between 09:00 and 10:00 a.m.) when mice were in the fed state. Blood was collected via submandibular bleeding. Serum was separated by centrifugation at 9000 rpm, and the serum levels of TNFα were measured by ELISA (Affymetrix eBioscience, CA, USA).

2.4. Extraction of Arcuate Nucleus

Mice were sacrificed in the light cycle between 09:00 and 10:00 a.m., and the arcuate nucleus of the hypothalamus was rapidly dissected from 1.0-mm thick sagittal sections of fresh brain. The arcuate nucleus, the ventral part of the medial hypothalamus with anterior and dorsal margins (approximately 0.5 mm from the ventral surface of the medial hypothalamus) and posterior margin (border with the mammillary body), was dissected using an Alto Stainless Steel Sagittal 1.0 mm Brain Matrix (Roboz Surgical Instrument Co., MD, USA) and Sharp Matrix Blades (Kent Scientific Co., CT, USA) (Minokoshi et al., 2004). The disected arcuate nucleus was immediately frozen in liquid nitrogen until RNA extraction.

2.5. Brain Collection for Immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with a cold fixative containing 4% parafomaldehyde (PFA) in phosphate buffered saline (PBS) pH 7.4, between 09:00 and 10:00 a.m. in the fed state. After fixation, brains were removed and immersed in the same fixative for 2 h at 4 °C. The brains were kept in PBS containing 10–20% sucrose at 4 °C for cryoprotection. They were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) and stored at −80 °C until sectioning. Brains were cut into 20-μm sections on a cryostat at −20 °C, thawed and mounted on Superfrost Plus microscope slides (Matsunami, Tokyo, Japan), and stored at −80 °C until immunohistochemistry was performed as described previously (Ito et al., 2013).

2.6. Intracerebroventricular Injection of TNFα

After 12 h fasting, male WT and KO mice at the age of 10 weeks on a chow diet were deeply anesthetized and placed into a small animal stereotaxic instrument (KOPF, CA, USA). Mice were implanted with a Hamilton needle (2.0 μL syringe) into the right lateral ventricle of the brain (0.34 mm posterior to bregma, 1.0 mm lateral to the midline, and 2.6 mm below the surface of the skull). TNFα (10−12 M) or saline in a volume of 2.0 μL was injected into the lateral ventricle over 1 min. Fifteen min after injection, mice were transcardially perfused with a cold fixative containing 4% PFA in sterile PBS pH 7.4, and analyzed with immunohistochemistry. The correct position of the intracerebroventricular (icv) injection was confirmed under the microscope with Hamilton needle marks recognized on the 20 μm brain sections cutting by cryostat.

2.7. Intraperitoneal Injection of Leptin

In order to compare the signaling between TNFα and leptin, 10-week-old mice on a chow diet were employed for ip injection of leptin as well. Male WT and KO mice were fasted overnight (12 h) and injected intraperitoneal (ip) with mouse recombinant leptin (A.F. Parlow, National Hormone and Peptide Program [NHPP], Harbor–UCLA Medical Center, Torrance, California; 1 μg/g body weight) as described previously (Banno et al., 2010; Shibata et al., 2016). Forty-five min after injection, mice were anesthetized and perfused with 4% PFA in PBS, pH 7.4.

2.8. Hypothalamic organotypic cultures

Sixteen-day-old WT and KO mice were sacrificed by decapitation, and hypothalamic tissues were sectioned at 350 μm thicknesses on a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). Four coronal slices containing the arcuate nucleus were separated and placed in HBSS (Invitrogen, Grand Island, NY, USA) enriched with glucose. Explants from individual mice were placed on 0.4 μm Millicell–CM filter inserts (Millipore, Billerica, MA, USA), and each filter insert was placed in a Petri dish (35 mm) containing 1.1 mL of culture medium (75% Earle’s MEM, 25% HBSS, 25 U/ml penicillin/streptomycin). The culture was maintained for 48 h at 37 °C in 5% CO2–enriched air under stationary conditions and the medium was changed every 24 h. Incubation with TNFα, STAT3 inhibitors, liposomal clodronate or leptin was performed in each experiment. Total RNA and protein from the hypothalamic slices were extracted 72 h after starting the cultures and subjected to analyses with quantitative realtime PCR (qRT-PCR). Western blot or immunohistochemistry, as described previously (Ito et al., 2012; Adachi et al., 2014; Onoue et al., 2016).

2.9. Effects of TNFα on the mRNA Expression and the Phosphorylation of Signal-transducing Proteins Downstream of TNFα Receptor Signaling in Hypothalamic Slice Cultures

To examine the time-course of TNFα effects on mRNA expression levels of Ptpn1 and inflammatory cytokines (Tnf, Il10, Il6 and Il1b), hypothalamic slice cultures were incubated with 100 ng/mL TNFα (R&D systems, MN, USA) for 0.5, 1, 2, 3, 6 or 24 h, while control explants were incubated with a vehicle (sterile PBS) for 0.5 h, and total RNA and proteins were extracted. To examine the dose-response of TNFα on inflammatory cytokines, hypothalamic explants were incubated with vehicle...
or TNFα (25, 50 or 100 ng/mL) for 1 h. For the dose-response of TNFα on Ptpn1, hypothalamic explants were incubated for 3 h. To examine the effects of TNFα on phosphorylation of JAK2, STAT3, mitogen-activated protein kinase (MAPK) and NFκB p65, hypothalamic explants were incubated with TNFα (100 ng/mL) for 0.5 h, and total RNA and protein were extracted.

![Image of graphs and figures](image_url)
2.10. Effects of STAT3 Inhibitor on TNFα-induced Cytokine Expression in Hypothalamic Slice Cultures

In order to determine whether PTP1B-meditation of the expression of inflammatory cytokines occurred through the JAK2-STAT3 pathway, hypothalamic slices were incubated with two classes of STAT3 inhibitor, JSI-124 (0.5 or 5 μM; Sigma-Aldrich, MO, USA) for 4 h or S31-201 (30 or 100 μM; Santa Cruz Biotechnology, TX, USA) for 2 h. Dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) was used as the control. The slices were incubated with TNFα (100 ng/mL) or vehicle (sterile PBS) together with JSI-124, S31-201 or DMSO for an additional 0.5 h, and total RNA and protein were extracted.

2.11. Depleting Microglia from Hypothalamic Slice Cultures

After establishing the cultures (24 h), we incubated the hypothalamic slices with 1.0 mg/mL of liposomal clodronate (Clo), which reportedly depleted microglial cells without altering other cell types (Vinet et al., 2012; Valdearcos et al., 2014), or with control liposomes (Lip) (Cosmobio, Tokyo, Japan) for 24 h. Slices were then placed in fresh medium, and 24 h later slices were treated with TNFα (100 ng/mL) or vehicle (sterile PBS) for 0.5 h, and total RNA and protein were extracted. For the evaluation of Iba1, GFAP and NeuN expression in hypothalamic slice cultures, the slices were fixed with 4% formaldehyde in PBS for 30 min, washed twice in PBS and kept in PBS containing 10–20% sucrose at 4 °C for cryoprotection. They were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) and stored at −80 °C until sectioning. Slices were cut into 30-μm sections on a cryostat at −20 °C, thaw mounted on Superfrost Plus microscope slides (Matsunami, Tokyo, Japan), stored at −80 °C and analyzed with immunohistochemistry as described previously (Hagiwara et al., 2014; Sato et al., 2005).

2.12. Effects of Leptin on Cytokine and Ptpn1 Expression in Hypothalamic Slice Cultures

Hypothalamic slices were incubated in the absence or presence of 100 ng/mL TNFα and 10−7 M leptin for 0.5 h, and total RNA and protein were extracted. To examine the effects of leptin on Ptpn1 mRNA expression, hypothalamic explants were incubated with leptin (10−8 M, 10−7 M or 10−6 M) or PBS (vehicle) for 24 h.

2.13. Determination of mRNA Levels by qRT-PCR

Total RNA was extracted from samples using TRIzol (Invitrogen, CA, USA) and the RNEasy kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 100 ng total RNA using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The qRT-PCR reactions were carried out using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, USA) and the RNeasy kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 100 ng total RNA using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The qRT-PCR reactions were carried out, and relative mRNA expression levels were calculated using the comparative Ct method as described previously (Ito et al., 2013). The sequences of primers are described in Supplementary Table 1.

2.14. Determination of Protein Levels by Western Blot

Samples of hypothalamic slices were lyzed in 100 μL of a buffer containing 10 mM Tris (pH 7.4), 50 mM NaF, 150 mM NaCl, 0.1% SDS, 2 mM Na3VO4, 5 mM EDTA, 1% Triton X-100 (Sigma-Aldrich, MO, USA) 1% sodium deoxycholate minimum and 1% protease inhibitor mix (Roche, Stockholm, Sweden). After centrifuging the samples, protein concentrations in the supernatants were determined using a bicinchoninic acid kit (Sigma-Aldrich, MO, USA). Protein (5–10 μg per sample) was run on a 10% Bis–Tris gel (Invitrogen) and transferred onto PVDF membranes (Millipore, MA, USA). Blots were blocked for 1 h in TBS-T solution [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween] containing 5% skim milk or 5% bovine serum albumin (Wako, Osaka, Japan). Membranes were incubated with antibodies against STAT3 phosphorylated at Tyr705, phospho-ERK, phospho-JNK, phospho-p38MAPK or phospho-NF-κB p65 (Cell Signaling, MA, USA) or phospho-JAK2 (Invitrogen) overnight at 4 °C. Primary antibodies were probed with HRP-conjugated donkey anti-rabbit IgG (NA934; GE Healthcare, Little Chalfont, UK) for 1 h at room temperature. To improve sensitivity and signal-to-noise ratio, Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan) was used for the dilution of the primary and secondary antibodies. Immunoreactivity was detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare). The membranes were stripped and incubated with antibodies against each nonphosphorylated protein or β-actin antibody (Abcam, Cambridge, UK) for normalization.

2.15. Immunohistochemistry

For detection of TNFα, the frozen sections were washed with PBS, 0.3% Triton X-100 in PBS (15 min) and 50 mM glycerine (15 min) followed by blocking with a mixture of 3% bovine serum albumin (Wako, Osaka, Japan) in PBS for 1 h at room temperature. Next, the sections were incubated with anti-TNFα antibody (1:50; R&D systems, MN, USA) overnight at 4 °C. The sections were then treated with Alexa Fluor 594-conjugated anti-goat IgG secondary antibody (1:500; Invitrogen) for 1 h at room temperature. After washing in 1× PBS, sections were placed on slides, air dried, and cover slipped with Vecashield (Vector Labs, CA, USA).

Immunohistochemistry of STAT3 phosphorylation was performed as described previously (Banno et al., 2010). Sections were washed with 1× PBS prior to and between successive blocking steps with 1.0% H2O2 / 1% NaOH in H2O (20 min), 0.3% glycerine (10 min), 0.03% SDS (10 min) and finally 0.2% sodium azide/ 3% normal goat serum / 0.25% Triton X-100 in PBS (1 h) at room temperature. Sections were then incubated with anti-p-STAT3 antibody (1:100; Cell Signaling, MA, USA), anti-iba1 (1:100, Wako, Osaka, Japan), anti-GFAP (1:100; Sigma-Aldrich, MO, USA) or anti-NeuN (1:100; Merck Millipore, Darmstadt, Germany) and then diluted in azide blocking solution overnight at 4 °C. The following day, sections were washed with 1× PBS and subsequently in sodium azide–free blocking solution containing Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (1:500; Invitrogen), Alexa Fluor 594-conjugated anti-mouse IgG secondary antibody (1:500; Invitrogen) or Alexa Fluor 647-conjugated anti-chicken IgY secondary antibody (1:500; Invitrogen) for 1 h at room temperature. After washing in 1× PBS, sections were placed on slides, air dried, and cover slipped with Vectashield (Vector Labs, CA, USA).

Fig. 1. Hypothalamic inflammation is decreased and p-STAT3-positive microglia are increased in PTP1B KO mice on a HFD. Body weight (a), epididymal fat pad weight (b) and serum TNFα (c) in PTP1B−/− (WT) and PTP1B−/− (KO) mice at the age of 7 weeks on a chow diet or a HFD. (d-f) The mRNA expression levels of Tnf, Il10, Il1b, Il6, Ptpn1, Iba1, Cd68, Adgre1, Gfap, Tubb3, Pomc and Agrp in the arcuate nucleus or the hypothalamus nucleus were assessed by qRT-PCR using Gapdh as an internal control. The qRTP-PCR reactions were carried out, and relative mRNA expression levels were calculated using the comparative Ct method as described previously (Ito et al., 2013). The sequences of primers are described in Supplementary Table 1.
Immunohistochemistry of PTP1B was performed as described previously (Dodd et al., 2015). Sections were subjected to antigen retrieval in citrate acid buffer [10 mM sodium citrate, 0.05% (v/v) Tween 20, pH 6.0] at 95 °C for 20 min. Sections were incubated at room temperature for 1 h in blocking buffer [0.1 M phosphate buffer, 0.2% (v/v) Triton X-100, 10% (v/v) bovine serum albumin (Wako, Osaka, Japan)] and then overnight at 4 °C with anti-PTP1B antibody (1:200; R&D systems, MN, USA), anti-Iba1 antibody (1:100; Wako Osaka, Japan), anti-GFAP (1:100; Sigma-Alrich, MO, USA) or anti-NeuN antibody (1:200; Merck Millipore, Darmstadt, Germany) in blocking buffer. After washing with PBS, sections were incubated with Alexa Fluor 488-conjugated anti-goat IgG secondary antibody (1:500; Invitrogen), Alexa Fluor 594-conjugated anti-rabbit IgG secondary antibody (1:500; Invitrogen), Alexa Fluor 594-conjugated anti-mouse IgG secondary antibody (1:500; Invitrogen) or DyLight 650-conjugated anti-chicken IgY secondary antibody (1:500; Abcam, Cambridge, UK) in blocking buffer for 2 h at room temperature. After washing in 1× PBS, sections were placed on slides, air dried, and cover slipped with Vectashield (Vector Labs).

All fluorescently stained sections were examined with a confocal laser microscope (TieA1R; NIKON INSTECH, Tokyo, Japan) and viewed using NIS-Elements software (NIKON INSTECH, Tokyo, Japan). Cells labeled for p-STAT3 were counted bilaterally in a blinded fashion. For analysis, we employed 4–6 mice in each group for the staining, and the mean values of 2 or 3 serial sections from each mouse were calculated. The sections included the arcuate nucleus (−1.70 mm to −1.82 mm from bregma based on coordinates in the brain atlas (Paxinos and Franklin, 2000]). The sample numbers in figure legends indicate the number of animals analyzed.

2.16. Statistical Analysis

The statistical significance of the differences between groups was analyzed by either unpaired t-test, one-way ANOVA, two-way ANOVA or two-way ANOVA with repeated measures followed by Bonferroni’s test by using SPSS Statistics 23 (IBM, NY, USA). Results are expressed as means ± SEM, and differences were considered significant at P < 0.05.

3. Results

3.1. Hypothalamic Inflammation Induced by HFD is Decreased in PTP1B KO Mice

Both body weight and epididymal fat pad weight of male mice at the age of 7 weeks were significantly higher on a HFD compared to those of mice on a chow diet in both PTP1B+/+ (WT) and PTP1B−/− (KO) mice (Fig. 1a, b). While body weights were significantly lower in KO compared to WT mice on a HFD after the age of 8 weeks (Supplementary Fig. 1a), there were no significant differences in body weights and epididymal fat pad weights between genotypes at the age of 7 weeks (Fig. 1a, b). There were no significant differences in serum TNFα levels between mice on a HFD and those on a chow diet, as well as between genotypes (Fig. 1c). On the other hand, tumor necrosis factor (Tnf) mRNA expression levels in the arcuate nucleus were significantly increased on a HFD compared with chow, but not in KO mice (Fig. 1d). The analysis of immunofluorescence staining also showed that TNFα immunoreactivity in the arcuate nucleus was increased on a HFD compared with chow in WT, but not in KO mice (Fig. 1e). The interleukin-10 (Il10) mRNA expression levels were significantly increased in WT mice on a HFD compared with chow, and the expression levels were further enhanced in KO mice (Fig. 1f). As reported previously (Zabolotny et al., 2008), the mRNA expression levels of the gene for PTP1B (Ptpn1) in the arcuate nucleus were significantly increased in WT mice on a HFD compared with chow (Supplementary Fig. 1b). The mRNA expression levels of interleukin-6 (Il6) and interleukin-1β (Il1b) in the arcuate nucleus were significantly increased on a HFD
compared with chow, but there were no significant differences in the expression levels between genotypes (Fig. 1f and Supplementary Fig. 1c).

3.2. PTP1B Immunoreactivity in the Arcuate Nucleus

To examine the localization of PTP1B immunoreactivity in the arcuate nucleus, ionized calcium binding adaptor molecule 1 (Iba1), glial fibrillary acidic protein (GFAP) and neuronal nuclei (NeuN) were used as markers of microglia, astrocytes and neurons, respectively. Double immunostaining revealed that PTP1B-immunoreactivity was observed in the microglia, astrocytes and neurons in WT mice (Supplementary Fig. 1d), whereas no immunoreactivity was observed in KO mice (data not shown).

3.3. HFD Induces the Phosphorylation of STAT3 in Microglia in the Arcuate Nucleus

Signal transducer and activator of transcription 3 (STAT3) phosphorylation in the arcuate nucleus was significantly increased in mice on a HFD compared to chow; there were no significant differences in STAT3 phosphorylation between genotypes (Fig. 1h, j). Double-immunostaining revealed that phospho-STAT3-positive cell numbers were significantly increased in microglia and astrocytes in KO mice compared to WT mice (Fig. 1i, k and Supplementary Fig. 1e, g). In contrast, there were no significant differences in the numbers of phospho-STAT3-positive neurons between genotypes (Supplementary Fig. 1f, h). Similar results were obtained when the percentages of microglia, astrocytes or neurons in pSTAT3-positive cells in the arcuate nucleus were analyzed (Supplementary Table 2).

3.4. TNFα Induces Phospho-STAT3 in Microglia But Not in Astrocytes or Neurons in the Arcuate Nucleus in KO Mice

To evaluate whether TNFα induces hypothalamic STAT3 phosphorylation, we conducted icv injection of 10−12 M TNFα. This dose of TNFα did not cause any significant changes in STAT3 phosphorylation in WT mice, whereas STAT3 phosphorylation was significantly increased in the arcuate nucleus in PTP1B KO mice (Fig. 2a, c). The anatomical analysis revealed that phospho-STAT3-positive cell numbers were significantly increased in microglia after TNFα injection in KO compared to WT mice (Fig. 2b, d), whereas there were no significant differences in cell numbers between genotypes in astrocytes and neurons (Supplementary Fig. 2 and Supplementary Table 3).

3.5. Leptin Induces Phospho-STAT3 in Astrocytes and Neurons But Not in Microglia in the Hypothalamus

While ip injection of leptin induced STAT3 phosphorylation in the arcuate nucleus in both WT and KO mice, the phosphorylation was significantly increased in KO mice compared to WT mice (Fig. 3a, c). The phospho-STAT3-positive cell numbers were significantly increased after leptin injection in both astrocytes and neurons in KO compared to WT mice (Supplementary Fig. 3). In contrast, leptin did not induce STAT3 phosphorylation in microglia in WT or KO mice (Fig. 3b, d and Supplementary Table 4).

3.6. PTP1B-deficiency Attenuates Inflammation Induced by TNFα in Hypothalamic Cultures

The incubation of hypothalamic explants with TNFα significantly increased Ptpn1 mRNA expression levels (Fig. 4a, b). Incubation with TNFα significantly increased Tnf and Il10 mRNA expression levels in both genotypes (Fig. 4c-f). In contrast, the increased Tnf mRNA expression levels were significantly attenuated (Fig. 4c, d) and Il10 mRNA expression levels were significantly attenuated (Fig. 3a, c) in KO mice compared to WT mice after leptin injection in both genotypes (Supplementary Table 3).

**Fig. 3.** Leptin induces STAT3 phosphorylation in astrocytes and neurons but not in microglia in the hypothalamus. (a and c) Immunostaining of p-STAT3 cells (a) and the p-STAT3-positive cell numbers (c) in the arcuate nucleus after ip injection of saline or 1 μg/g leptin, and the p-STAT3-positive cell numbers in microglia (d) in KO mice (n = 4). All values are means ± SEM. Statistical analyses were performed by either two-way ANOVA (c) or unpaired t-test (d). *, p < 0.05 versus saline in WT. †, p < 0.05 versus saline in KO. NS, not significant. Scale bar = 20 μm. 3 V: third ventricle.
levels were significantly increased (Fig. 4e, f) in KO mice compared to WT mice. The increased Il1b mRNA expression levels were also significantly attenuated in KO mice compared to WT mice (Supplementary Fig. 4a, b), whereas the Il6 mRNA expression levels were similar between genotypes (Fig. 4g, h). Incubation with TNFα increased the phosphorylation of both JAK2 and STAT3 in hypothalamic explants in both genotypes. In contrast, phosphorylation was increased to a greater extent in KO mice (Fig. 4i, j). Phosphorylation of other signal-transducing proteins such as p65, p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) was increased similarly in response to TNFα in both genotypes (Fig. 4k and Supplementary Fig. 4c-e).

3.7. Inhibition of the JAK2-STAT3 Pathway Cancels the Differences of Inflammation Between Genotypes

In order to determine whether the attenuation of hypothalamic inflammation by PTP1B-deficiency was mediated through the JAK2-STAT3 pathway, we treated hypothalamic explants with two classes of STAT3 inhibitors, JSI-124 and S31-201. We confirmed that STAT3 inhibitors decreased STAT3 phosphorylation in a dose-dependent manner (Fig. 5a, b), and that the STAT3 phosphorylation induced by TNFα was decreased to similar levels in both WT and KO mice by the inhibitors (Fig. 5c, d). On the other hand, STAT3 inhibitors did not affect the phosphorylation of other signal-transducing proteins such as p65, p38, JNK and ERK (Supplementary Fig. 5a–d and 5f–i). Incubation with the inhibitors of STAT3 phosphorylation completely inhibited the downregulation of Tnf and upregulation of Il10 mRNA expression in KO mice treated with TNFα (Fig. 5e-h). Incubation with the inhibitors of STAT3 phosphorylation also blocked the downregulation of Il1b (Supplementary Fig. 5e, j).

3.8. Microglial Depletion Abolishes the Attenuation of Inflammation by PTP1B-Deficiency

We used liposomal clodronate (Clo) to deplete microglia from the hypothalamic slice cultures; liposomes (Lip) were used as controls. Microglial depletion was confirmed by a marked reduction in the mRNA expression levels of microglial markers such as allograft inflammatory factor 1 (Aif1), cluster of differentiation 68 (Cd68), and G protein-coupled receptor E1 (Adgre1) (Fig. 6a). In addition, we also confirmed the loss of Iba1-positive cells by immunofluorescence staining (Fig. 6b). Treatment with Clo did not change mRNA expression levels of Gfap, tubulin, beta 3 class III (Tubb3), Pomc or agouti-related protein (Agrp) compared to control (Fig. 6a). The expression levels of GFAP or NeuN proteins in immunofluorescently-positively cells were not affected by Clo treatment (Fig. 6b). Microglial depletion remarkably decreased the mRNA expression levels of Tnf and Il10 compared to controls, and completely blocked the downregulation of Tnf and upregulation of Il10 mRNA expression by TNFα in KO mice (Fig. 6c, d).

3.9. STAT3 Phosphorylation Induced by Leptin Does Not Affect Hypothalamic Inflammation Induced by TNFα

Incubation with leptin induced STAT3 phosphorylation in the hypothalamic cultures (Fig. 7a). On the other hand, leptin treatment did not affect the mRNA expression levels of Tnf, Il10 or Il1b after TNFα treatment (Fig. 7b, c and Supplementary Fig. 6), Incubation with 10^{-6} M but not 10^{-7} M or 10^{-8} M leptin significantly increased the Ptpn1 mRNA expression levels in hypothalamic cultures (Fig. 7d).

4. Discussion

In the present study, we demonstrated that hypothalamic inflammation induced by a HFD, as assessed by the expression levels of TNFα and IL-10, was significantly attenuated, and that STAT3 phosphorylation in microglia in the arcuate nucleus was more increased in PTP1B KO compared to WT mice. Using hypothalamic cultures, we showed that inflammation induced by TNFα was significantly attenuated, accompanied by increased phosphorylation of both JAK2 and STAT3, in KO compared to WT mice. In addition, incubation with inhibitors of STAT3 phosphorylation or microglial depletion eliminated the difference in inflammation between the genotypes. Furthermore, we found that injection of leptin increased STAT3 phosphorylation in hypothalamic neurons and astrocytes and significantly increased STAT3 phosphorylation in hypothalamic cultures, but it did not affect inflammation. Thus, our data suggest that the deficiency of PTP1B attenuated hypothalamic inflammation via the activation of the JAK2-STAT3 signaling pathway in microglial cells.

It is reported that a HFD reduces leptin-induced STAT3 phosphorylation in the arcuate nucleus of WT mice (Munzberg et al., 2004), and that leptin sensitivity in hypothalamic neurons was increased in PTP1B KO compared to WT mice, which makes KO mice resistant to diet-induced obesity (Zabolotny et al., 2002; Bence et al., 2006). In the present study, hypothalamic inflammation, which could be related to obesity and leptin resistance (De Git and Adan, 2015), was examined in mice at the age of 7 weeks when there were no significant differences in body weight between the WT and KO mice fed a HFD. Thus, the comparison between genotypes in the present study has provided us with an opportunity to elucidate the underlying mechanisms by which body weight is increased in WT compared to KO mice on a HFD.

Consistent with previous studies (De Souza et al., 2005; Valdecaros et al., 2014), our data showed that TNFα immunoreactivity was increased in the arcuate nucleus in WT mice maintained on a HFD, but this was not the case in PTP1B KO mice. The analyses of Tnf and Il10 mRNA expression levels further support the conclusion that inflammation in the hypothalamus was not activated in KO mice at the age of 7 weeks. Thus, it is indicated that hypothalamic inflammation preceded the relative increases in body weight in WT compared to KO mice fed a HFD. On the other hand, our data also showed that there were no significant differences in the activation of signaling pathways such as p65, p38, JNK and ERK between genotypes. This is probably because these signal transducers downstream from the TNFα receptor are not substrates for PTP1B (Tsou and Bence, 2012).

There are several lines of evidence that the JAK2-STAT3 signaling pathway functions downstream from the TNFα receptor (Romanatto et al., 2007; Guo et al., 1998; Miscia et al., 2002; Sica and Mantovani, 2012). The activated STAT3 is reported to constitute a dimer that translocates into the nucleus to exert anti-inflammatory effects by decreasing Tnf mRNA expression and increasing Il10 mRNA expression (Benkhart et al., 2000; Hutchins et al., 2012, 2013). Conversely, STAT3 deficiency in macrophages is reported to increase inflammation (Takeda et al., 1999; Yoo et al., 2002), and previous studies revealed that PTP1B deficiency in macrophages decreased TNFα expression and increased IL-
a. STAT3 and p-STAT3 levels under TNFα, JSI-124, and S31-201 treatments.

b. STAT3 and p-STAT3 levels under TNFα, JSI-124, and S31-201 treatments.

c. STAT3 and p-STAT3 levels under TNFα, JSI-124, and S31-201 treatments.

d. STAT3 and p-STAT3 levels under TNFα, JSI-124, and S31-201 treatments.

e. Tnf mRNA expression levels under TNFα, JSI-124, and S31-201 treatments.

f. Tnf mRNA expression levels under TNFα, JSI-124, and S31-201 treatments.

.g. Il10 mRNA expression levels under TNFα, JSI-124, and S31-201 treatments.

h. Il10 mRNA expression levels under TNFα, JSI-124, and S31-201 treatments.
10 expression via activation of the STAT3 pathway in the peripheral tissues (Zhang et al., 2013; Grant et al., 2014; Pike et al., 2014). In the present study, we confirmed the findings of previous studies that STAT3 phosphorylation in the hypothalamus was increased in WT mice on a HFD (Ottaway et al., 2015; Knight et al., 2010; Martin et al., 2006). Our data also demonstrated that STAT3 phosphorylation in microglia increases more in KO mice than in WT mice on a HFD. The increased STAT3 phosphorylation is accompanied by the attenuation of hypothalamic inflammation in vivo and in primary hypothalamic cultures, and the inhibition of STAT3 phosphorylation as well as depletion of microglia completely eliminated the differences between genotypes in the cultures. These data suggest that PTP1B–deficiency in microglia activates the JAK2-STAT3 signaling pathways that lead to the attenuation of hypothalamic inflammation. Our data thus revealed an important role of the JAK2-STAT3 signaling pathway in hypothalamic microglia, a pathway that prevents hypothalamic inflammation and possibly obesity by a HFD. On the other hand, our data also showed that in WT mice, in which STAT3 phosphorylation is relatively low compared to KO mice, the decreased STAT3 phosphorylation by STAT3 inhibitors did not affect the inflammatory responses to the TNFα treatment (Fig.5 c-h), suggesting that the anti-inflammatory action of the JAK2-STAT3 pathway manifests only after the phosphorylation reaches certain levels.

In the present study, in vivo leptin treatment induced the phosphorylation of STAT3 in neurons and astrocytes but not in microglia, whereas it did not affect inflammation induced by TNFα in hypothalamic cultures (Pan et al., 2008; Wang et al., 2015). Consistent with our findings, it is demonstrated that leptin receptors are not expressed in microglia in mice (Pan et al., 2008). On the other hand, there are also reports showing that leptin phosphorylated STAT3 in microglial cells in rats or the BV-2 cell line (Tang et al., 2007; Pinteaux et al., 2007). While further studies are required to explain the discrepancies among studies, our findings clearly indicate that the JAK2-STAT3 pathways have different roles among neurons and microglia: in the former, they mediate leptin signaling and in the latter they mediate signaling of cytokines such as TNFα. The graphical abstract shows schematic illustrations of the roles of the JAK2-STAT3 pathways in neurons and microglia, and in both, PTP1B negatively regulates the pathways.

Our data showed that TNFα and leptin treatment in hypothalamic cultures increased Ptpn1 mRNA expression. The increased PTP1B could potentially decrease STAT3 phosphorylation and therefore activate inflammation in microglia, although the increases in Ptpn1 expression were relatively small in the present study.

As TNFα transduces inflammatory signals in various tissues, including the hypothalamus (Purkayastha et al., 2011; De Git and Adan, 2015), it is plausible that TNFα in the hypothalamus induced by a HFD mediates the inflammation, at least partially. We therefore employed TNFα treatment and several inflammatory cytokines were indeed induced by the treatment in the present study. However, it is also possible that other inflammatory cytokines such as IL-1β and IL-6 are also induced by a HFD (Thaler et al., 2012; De Souza et al., 2005) and act on hypothalamic microglia to induce inflammation. Furthermore, other molecules downstream from the TNFα receptor that were not examined in this study might also play a role in hypothalamic inflammation. Our data also showed that STAT3 phosphorylation was increased not only in microglia but also in astrocytes in KO mice fed a HFD. Given that not only microglia but also astrocytes and neurons reportedly release inflammatory cytokines (Gupta et al., 2012; Wellhauser and Belsham, 2014), it is likely that there are some communications among these cells, and further studies are necessary to clarify the roles of microglia, astrocytes and neurons in hypothalamic inflammation.
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