Biochemical and Biophysical Research Communications 488 (2017) 116–121

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

PTP1B deficiency improves hypothalamic insulin sensitivity resulting in the attenuation of *AgRP* mRNA expression under high-fat diet conditions

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ARTICLE INFO

Article history:
Received 25 April 2017
Accepted 3 May 2017
Available online 4 May 2017

Keywords:
PTP1B
Insulin
Hypothalamus
AgRP

ABSTRACT

Hypothalamic insulin receptor signaling regulates energy balance and glucose homeostasis via agouti-related protein (AgRP). While protein tyrosine phosphatase 1B (PTP1B) is classically known to be a negative regulator of peripheral insulin signaling by dephosphorylating both insulin receptor β (IRβ) and insulin receptor substrate, the role of PTP1B in hypothalamic insulin signaling remains to be fully elucidated. In the present study, we investigated the role of PTP1B in hypothalamic insulin signaling using PTP1B deficient (KO) mice *in vivo* and *ex vivo*. For the *in vivo* study, hypothalamic insulin resistance induced by a high-fat diet (HFD) improved in KO mice compared to wild-type (WT) mice. Hypothalamic *AgRP* mRNA expression levels were also significantly decreased in KO mice independent of body weight changes. In an *ex vivo* study using hypothalamic organotypic cultures, insulin treatment significantly increased the phosphorylation of both IRβ and Akt in the hypothalamus of KO mice compared to WT mice, and also significantly decreased *AgRP* mRNA expression levels in KO mice. While incubation with inhibitors of phosphatidylinositol-3 kinase (PI3K) had no effect on basal levels of Akt phosphorylation, these suppressed insulin induction of Akt phosphorylation to almost basal levels in WT and KO mice. The inhibition of the PI3K-Akt pathway blocked the downregulation of *AgRP* mRNA expression in KO mice treated with insulin. These data suggest that PTP1B acts on the hypothalamic insulin signaling via the PI3K-Akt pathway. Together, our results suggest a deficiency of PTP1B improves hypothalamic insulin sensitivity resulting in the attenuation of *AgRP* mRNA expression under HFD conditions.

1. Introduction

Insulin plays a critical role in the regulation of glucose homeostasis via direct actions on insulin receptors expressed in muscle, liver and adipocytes [1]. Since insulin receptor mRNA is expressed in the brain [2], insulin also regulates glucose homeostasis via the central nervous system [3]. Of note, a previous study revealed that insulin receptor signaling via agouti-related protein (AgRP), an orexigenic neuropeptide, in the hypothalamic arcuate nucleus regulates hepatic glucose production via vagus nerves [3–6]. Central insulin receptor signaling is also well known to play an important role in regulating energy balance [7]. Energy balance is regulated by specific regions of the central nervous system, including the hypothalamus, and the sum of energy intake and energy expenditure defines body weight [8]. The activation of AgRP neurons induces feeding, reduces energy expenditure and ultimately increases body weight [9]. Activated insulin receptors phosphorylate the insulin receptor substrate (IRS) proteins, which

Abbreviations: HFD, high-fat diet; AgRP, agouti-related protein; PTP1B, protein tyrosine phosphatase 1B; IRβ, insulin receptor β; IRS, insulin receptor substrate; icv, intracerebroventricular; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; ip, intraperitoneally; qRT-PCR, quantitative real-time PCR; FoxO, forkhead box-containing protein of the O subfamily; PI3K, phosphatidylinositol-3 kinase; PDK1, phosphatidylinositol-3-OH kinase/3-phosphoinositide-dependent protein kinase 1.

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http://dx.doi.org/10.1016/j.bbrc.2017.05.019
0006-291X/

then activate phosphatidylinositol-3 kinase (PI3K) leading to the activation of phosphatidylinositol-3-OH kinase/3-phosphoinositide-dependent protein kinase 1 (PDK1) [10]. A previous study revealed that AgRP neuron-specific PDK1 deficient mice showed a reduction in food intake and body weight [11]. Thus, insulin regulates glucose homeostasis and energy balance via AgRP neurons in the hypothalamus, at least in part.

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitous non-transmembrane member of the protein tyrosine phosphatase family that is encoded by the *Ptpn1* gene [12]. PTP1B is widely expressed in the body, particularly in the brain where PTP1B is found in high concentration in the hypothalamic arcuate nucleus [13]. Whole body, brain or pro-opiomelanocortin (POMC) neuron-specific PTP1B deficiency was reported to protect against high-fat induced obesity and impairment of glucose metabolism [14–16]. While PTP1B is classically known to be a negative regulator of peripheral insulin signaling by dephosphorylating both the insulin receptor β (IRβ), IRS-1 and IRS-2, the role of PTP1B in central insulin signaling remains to be fully elucidated [17]. In support of a relationship between central insulin signaling and PTP1B in the hypothalamus, intracerebroventricular (icv) injection of insulin was found to improve insulin signaling in rats when the expression level of hypothalamic PTP1B was partially reduced by icv injection of PTP1B antisense oligonucleotides [18]. These approaches, however, do not exclude the possibility that insulin acts outside the hypothalamus in this content, and it remains unclear whether hypothalamic PTP1B can directly regulate insulin signaling. Moreover it remains to be seen if the activation of hypothalamic insulin signaling due to a deficiency of PTP1B affects the expression of feeding-related neuropeptides such as AgRP, neuropeptide Y (NPY) and POMC.

In the present study, we investigated the role of PTP1B in the hypothalamic insulin signaling using PTP1B deficient mice *in vivo* and *ex vivo*. *In vivo* study showed that insulin resistance in the hypothalamic arcuate nucleus induced by a HFD improved and that *AgRP* mRNA expression was decreased in PTP1B deficient mice independent of body weight changes. *Ex vivo* study using hypothalamic organotypic cultures showed that insulin treatment increased insulin sensitivity and decreased *AgRP* mRNA expression in PTP1B deficient mice via the activation of the PI3K-Akt pathway downstream from insulin receptor signaling. Our results suggest that PTP1B deficiency improves hypothalamic insulin sensitivity resulting in the attenuation of *AgRP* mRNA expression under HFD conditions.

2. Materials and methods

2.1. Animals

PTP1B$^{-/-}$ mice [12,13] 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 National Institutes of Health animal care guidelines. Mice were maintained with a 12 h light/12 h dark cycle in a temperature-controlled barrier facility, and 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 of age), male mice were placed on diets of either standard chow (CE-2; calories provided by protein [24.9%], fat [4.6%], and carbohydrate [70.5%]; CLEA Japan, Tokyo, Japan) or a custom high-fat diet (HFD 58Y1; calories provided by protein [18.3%], fat [60.9%], and carbohydrate [20.1%]; TestDiet, MO, USA). Body weight was monitored until the age of 16 weeks.

2.3. Intraperitoneal injection of insulin

For insulin signaling experiments, animals were fasted for 8 h. Saline or insulin (10 mU/g body weight) was then injected intraperitoneally (ip) into male WT and KO mice (7 weeks of age) on a chow diet or HFD, and arcuate nucleus were removed 15 min later. Blood glucose was measured in tail blood using a glucometer (Medi-safe mini; Terumo, Tokyo, Japan) before and after saline or insulin administration.

2.4. Sampling in vivo

Mice were decapitated during the light cycle between 09:00 and 10:00 a.m. After decapitation, the brain was immediately removed and the arcuate nucleus of the hypothalamus was dissected from 1.0-mm thick sagittal sections of fresh brain at the ages of 4 and 7 weeks. The arcuate nucleus was dissected as described previously [19]. The dissected arcuate nucleus was immediately frozen in liquid nitrogen until RNA extraction. The measurement of body weight and epididymal fat pad weight were also performed at 7 weeks of age.

2.5. Hypothalamic organotypic cultures

Sixteen-day-old WT and KO mice were sacrificed by decapitation, and cultured four hypothalamic slices containing the arcuate nucleus were performed as described previously [19]. Incubation with insulin or PI3K inhibitors was performed in each experiment. Total RNA and protein from hypothalamic slices were extracted 72 h after starting the cultures and subjected to analyses with quantitative real-time PCR (qRT-PCR) or western blot.

2.6. Effects of PTP1B on insulin signal transduction in hypothalamic slice cultures

To examine the effects of insulin on phosphorylation of both IRβ and Akt in hypothalamic organotypic cultures, hypothalamic slices were treated with insulin (10^{-7} M) or control medium for 5 min and protein was extracted. To evaluate *AgRP, NPY* and *POMC* mRNAs, hypothalamic slices were treated with insulin (10^{-7} M) or control medium for 24 h and total RNA was extracted.

2.7. Effects of PI3K inhibitor on the mRNA expression levels of AgRP in hypothalamic slice cultures treated with insulin

In order to evaluate whether PTP1B regulated *AgRP* mRNA expression via the PI3K-Akt pathway, hypothalamic slices were incubated with two classes of PI3K inhibitor, wortmannin (10^{-7} M; Selleckchem, TX, USA) or LY294002 (10^{-5} M; Selleckchem), each for 4 h. Dimethyl sulfoxide (DMSO; Wako, Osaka, Japan) was used as the control. The slices were then incubated with insulin (10^{-7} M) or vehicle (sterile PBS) together with wortmannin, LY294002 or DMSO for an additional 5 min for protein extraction, and 24 h for total RNA extraction.

2.8. Measurement of serum insulin levels

Blood was collected from the tail at the age of 4 or 7 weeks at the beginning of the light cycle (between 09:00 and 10:00 a.m.) when mice were in a fed state. Serum was separated by centrifugation at 6000 rpm, and serum levels of insulin were measured by ELISA

(Morinaga Institute of Biological Science, Kanagawa, Japan).

2.9. Determination of mRNA levels by qRT-PCR

Total RNA was extracted from samples and qRT-PCR was performed as described previously [20]. The relative mRNA levels of *NPY*, *Pomc* and *Agrp* in arcuate nucleus or hypothalamic slice explants were assessed by qRT-PCR as described previously [20].

2.10. Determination of protein levels by western blot

Samples of hypothalamic slices were lysed in 100 μL of a buffer containing 10 mM Tris (pH 7.4), 50 mM NaF, 150 mM NaCl, 0.1% SDS, 2 mM Na_3VO_4, 5 mM EDTA, 1% Triton X-100 (Sigma–Aldrich, MO, USA), and a minimum 1% sodium deoxycholate and 1% protease inhibitor mix (Roche, Stockholm, Sweden). Western blotting was performed as described previously [19]. Membranes were incubated with phosphorylated IRβ and Akt antibody (Cell Signaling, MA, USA). The protein expression levels were normalized to each non-phosphorylated protein antibody. Blot was quantified using NIH ImageJ software.

2.11. Statistical analysis

The statistical significance of the differences between groups was analyzed by either unpaired *t*-tests or by one-way or two-way ANOVA with repeated measures followed by Bonferroni's test as appropriate. Results are expressed as mean ± standard error of the mean (SEM) and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Insulin resistance in the hypothalamic arcuate nucleus induced by a HFD is improved in KO mice

Firstly, we evaluated insulin signaling in the hypothalamic arcuate nucleus by evaluation of the phosphorylation of IRβ and Akt after 15 min of an intraperitoneal (ip) insulin injection to mice fasted for 8 h. We confirmed that an ip injection of insulin (1, 5, 10 mU/gBW) induced the phosphorylation of both IRβ and Akt in WT mice on a chow diet (Fig. 1A and B). We also confirmed that ip injection of insulin did not cause hypoglycemia in mice after 15 min of administration. Next we evaluated insulin signaling in mice on a chow diet or a HFD using animals at the age of 7 weeks when there were no significant differences in body weights or epididymal fat pad weights between genotypes (data not shown). In WT mice, the increased phosphorylation of both IRβ and Akt induced by insulin administration in mice on a chow diet was significantly more compared to that of animals on a HFD (Fig. 1C and D). By contrast, in KO mice, there were no significant differences in the phosphorylation of IRβ and Akt in animals on a HFD compared to those on a chow diet (Fig. 1E and F).

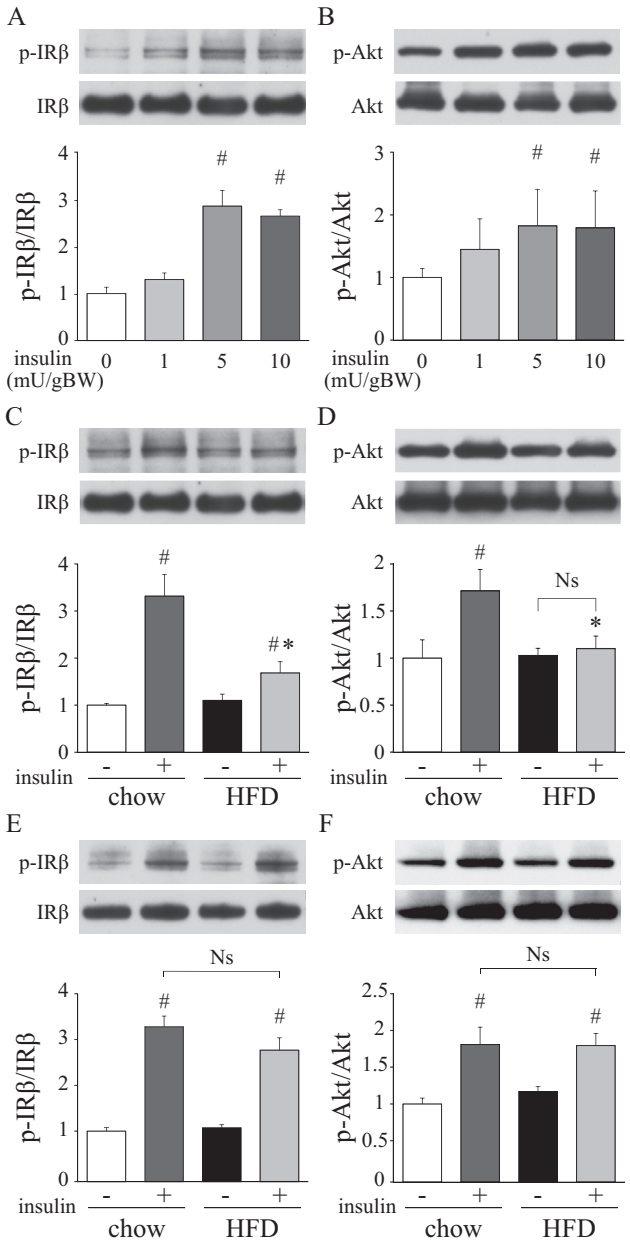

Fig. 1. Insulin resistance in the hypothalamic arcuate nucleus induced by a HFD is improved in mice.
(A) IRβ and (B) Akt phosphorylation in the hypothalamic arcuate in WT mice after ip injection of saline or insulin (1, 5, 10 mU/gBW). (C) IRβ phosphorylation and (D) Akt phosphorylation in the arcuate nucleus in WT mice on a chow diet or a HFD after an ip injection of saline or insulin (10 mU/gBW). (E) IRβ and (F) Akt phosphorylation in the arcuate nucleus in KO mice on a chow diet or HFD after ip injection of saline or insulin (10 mU/gBW). Levels of each phosphoprotein were determined by western blot and normalized to IRβ or Akt. All values are mean ± SEM. $^{*}p < 0.05$ versus chow. $^{\#}p < 0.05$ versus untreated. Ns, not significant.

3.2. Insulin decreased the mRNA expression of AgRP in KO mice under HFD conditions

To determine the effects of insulin on the expressions of feeding-related neuropeptides, we evaluated the mRNA expressions of *AgRP*, *NPY* and *POMC* in animals on a HFD at the age of 4 weeks. While body weight and blood glucose levels at the age were similar in WT and KO mice on a HFD (Fig. 2A and B), serum insulin levels were significantly decreased in KO mice compared to WT mice (Fig. 2C). Although insulin levels were low, *AgRP* mRNA expression levels in the arcuate nucleus were significantly decreased in KO mice compared to WT mice (Fig. 2D). However, significant differences in the mRNA expression of *NPY* or *POMC* were not noted between genotypes (Fig. 2E and F) at the age of 4 weeks.

3.3. The mRNA expression levels of AgRP were decreased in KO mice treated with insulin in hypothalamic slice cultures

In order to perform further investigation, we utilized

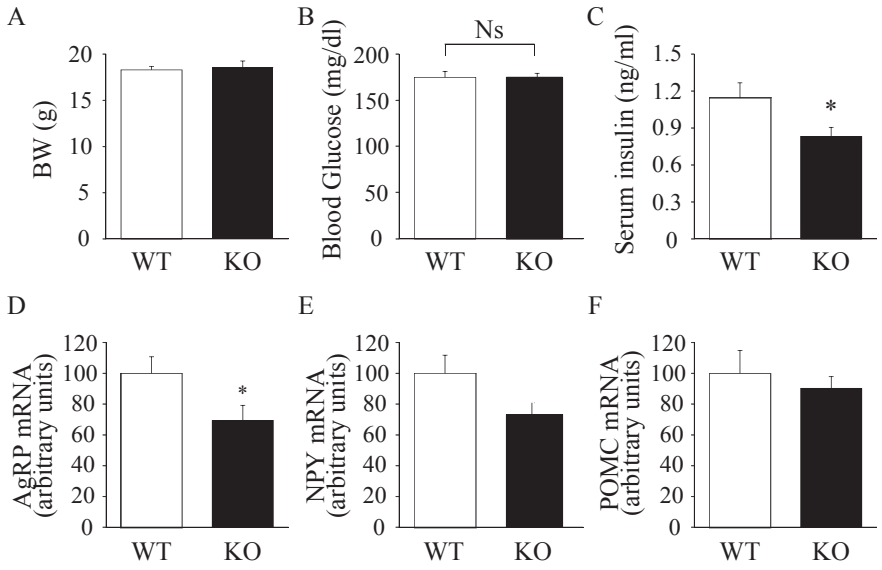

Fig. 2. Insulin decreased the mRNA expression of *AgRP* in KO mice under HFD conditions.
(A) Body weight, (B) blood glucose and (C) serum insulin in male WT and KO mice, aged 4 weeks on a HFD. The mRNA expression levels of (D) *AgRP*, (E) *NPY* and (F) *POMC* in the arcuate nucleus of male WT and KO mice, aged 4 weeks, on a HFD as assessed by qRT-PCR. All values are mean ± SEM. $^{*}p < 0.05$ versus WT. Ns, not significant.

hypothalamic primary cultures derived from WT and KO mice. First, we confirmed that incubation with insulin induced the phosphorylation of both IRβ and Akt in WT mice, and that such phosphorylations were significantly increased in KO mice compared to WT mice (Fig. 3A and B). Furthermore, we found that *AgRP* mRNA expression levels were significantly decreased in KO mice compared to WT mice (Fig. 3C). Significant differences in the mRNA expression levels of *NPY* or *POMC* were not noted between genotypes (Fig. 3D and E).

3.4. Inhibition of the PI3K-Akt pathway blocks the downregulation of AgRP mRNA expression in KO mice treated with insulin

To determine whether the mRNA expression of *AgRP* is mediated via the PI3K-Akt pathway, we treated hypothalamic cultures with either wortmannin or LY294002 in order to inhibit PI3K activity. While incubation with wortmannin had no effect on basal levels of Akt phosphorylation, it suppressed insulin induction of Akt phosphorylation to almost basal levels in WT and KO mice (Fig. 4A and B, respectively). The inhibition of the PI3K-Akt pathway by

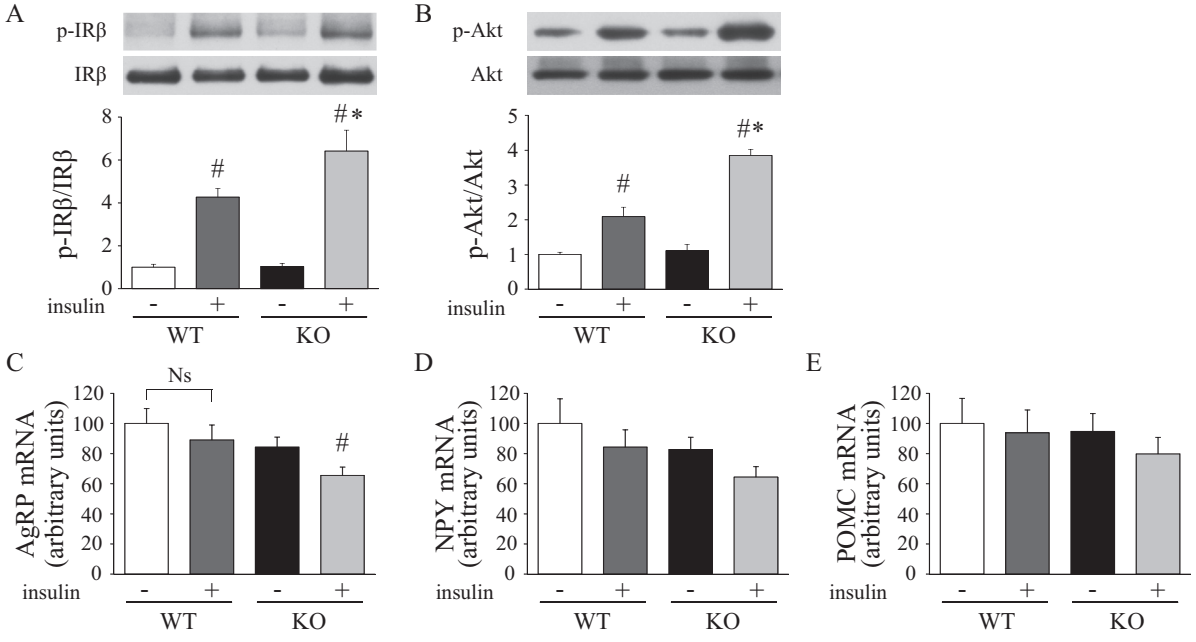

Fig. 3. *AgRP* mRNA in hypothalamic slice cultures was decreased in KO mice treated with insulin.
(A) IRβ and (B) Akt phosphorylation in hypothalamic organotypic cultures incubated with insulin (10^{-7}M) for 5 min as determined by western blot. (C) *AgRP*, (D) *NPY* and (E) *POMC* mRNA expression in hypothalamus incubated with insulin (10^{-7}M) for 24 h as assessed by qRT-PCR. Levels of each phosphoprotein were normalized to IRβ or Akt. All values are mean ± SEM. $^{*}p < 0.05$ versus WT. $^{\#}p < 0.05$ versus untreated. Ns, not significant.

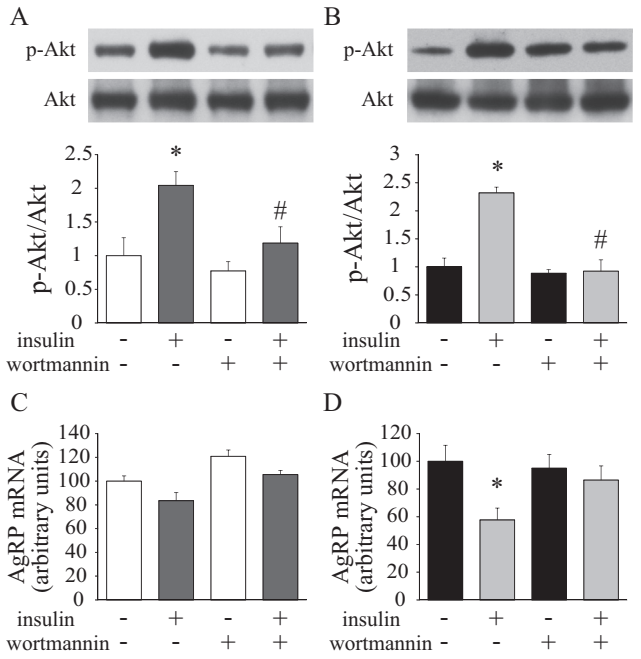

Fig. 4. Inhibition of the PI3K-Akt pathway blocks the downregulation of *AgRP* mRNA expression in KO mice treated with insulin.
Akt phosphorylation induced by insulin in the absence or presence of 10^{-7}M wortmannin in WT (A) and KO mice (B) as determined by western blot. Expression levels of *AgRP* mRNA in the absence or presence of 10^{-7}M wortmannin (C and D) in WT (A, C) and KO mice (B, D) as assessed by qRT-PCR. All values are mean ± SEM. $^{*}p < 0.05$ versus untreated. $^{\#}p < 0.05$ versus insulin.

wortmannin blocked the downregulation of *AgRP* mRNA expression in KO mice treated with insulin (Fig. 4D), while these PI3K inhibitors did not affect *AgRP* mRNA expression in WT mice (Fig. 4C). LY294002 showed the same data as wortmannin (data not shown).

4. Discussion

In the present study, we demonstrated that a HFD induced insulin resistance in the hypothalamic arcuate nucleus when compared to those on a chow diet in mice. The insulin resistance was improved in KO mice as shown by the increased phosphorylation of both IRβ and Akt. Furthermore, we found that *AgRP* mRNA levels decreased in KO mice via the activation of the PI3K-Akt pathway in hypothalamic slice cultures. These data suggest that (1) PTP1B is involved in the induction of insulin resistance in the hypothalamic arcuate nucleus under HFD conditions, (2) PTP1B negatively regulates insulin signaling by dephosphorylation of IRβ in the hypothalamus, and (3) enhanced hypothalamic insulin signaling due to a deficit of PTP1B attenuates mRNA expression levels of *AgRP*.

A previous study showed that *AgRP* mRNA expression was increased in *AgRP* neuron-specific insulin receptor (IR) deficient mice compared to WT mice, while *POMC* mRNA expression was unchanged in POMC neuron-specific IR deficient mice [5]. In addition, PTP1B deficiency was reported to enhance leptin but not insulin signaling in POMC neurons [21]. Taken together, our findings in the present study suggest that PTP1B plays an important role in the regulation of hypothalamic insulin signaling, specifically in AgRP neurons in the arcuate nucleus. In the present study, *AgRP* mRNA expression was significantly decreased in KO mice at the age of 4 weeks when body weight was similar between genotypes. Consistent with our data, the decreased mRNA expression levels of *AgRP* were confirmed in brain-specific PTP1B deficient mice [15]. In addition, when physiological conditions between genotypes in hypothalamic organotypic cultures were aligned, insulin certainly attenuated *AgRP* mRNA expression in KO mice via increased phosphorylation of both IRβ and Akt. The hypothalamic organotypic cultures employed in this study have been shown to maintain their intrinsic properties [22]. Our data in the slice cultures demonstrate that PTP1B directly regulates hypothalamic insulin signaling, and the activation of hypothalamic insulin signaling due to a deficiency of PTP1B affects the mRNA expression of *AgRP* without change of *NPY* or *POMC*. We also showed that inhibitors of PI3K canceled the downregulation of *AgRP* mRNA expression in KO mice, suggesting that PTP1B acts upstream of PI3K as well as in peripheral insulin signaling. A previous study reported that insulin attenuates hypothalamic *AgRP* mRNA expression by preventing the forkhead box-containing protein of the O subfamily (FoxO)-1, a transcriptional factor, from binding to the *AgRP* promoter [23]. Since FoxO-1 is phosphorylated and inactivated by Akt [24], it is plausible that the attenuation of *AgRP* mRNA expression in KO mice is due to the deactivation of FoxO-1 via an enhanced PI3K-Akt signaling pathway downstream from the insulin receptor, and the attenuation of *AgRP* mRNA expression prior to substantial weight gain contributes to the anti-obesity phenotype of PTP1B deficient mice under HFD conditions.

In the present study, we showed that the peripheral injection of insulin acts on the hypothalamic arcuate nucleus resulting in the increased phosphorylation of both IRβ and Akt *in vivo*. Previous papers showed that such phosphorylations were diminished under HFD conditions [25,26], which were consistent with our present data. Of note, the insulin resistance induced by HFD improved in KO mice independent of body weight changes. In this regard, we have previously reported that tumor necrosis factor-alpha, an inflammatory cytokine induced by HFD, increased hypothalamic PTP1B activity via the NFκB pathway [27]. Additionally, a deficiency of PTP1B attenuates hypothalamic inflammation via activation of the Janus Activating Kinase 2-Signal Transducers and Activator of Transcription 3 pathway in microglia [19]. Given that hypothalamic inflammation induced by a HFD is reported to induce hypothalamic insulin resistance independent of body weight changes [25], it is plausible that attenuated hypothalamic inflammation by PTP1B deficiency contributed to improve insulin sensitivity in KO mice under HFD conditions.

In conclusion, we demonstrated that a deficiency of PTP1B improved hypothalamic insulin sensitivity resulting in the attenuation of *AgRP* mRNA expression under HFD conditions.

Acknowledgements

We thank Michiko Yamada for helpful technical assistance. PTP1B$^{-/-}$ mice were thankfully provided by Dr. Benjamin Neel from the NYU School of Medicine in the USA.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.05.019.

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