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LMO4 is required to maintain hypothalamic insulin signaling

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ABSTRACT

Insulin action at the hypothalamus controls glucose homeostasis by suppressing hepatic glucose production and promoting glucose uptake by muscle. However, the mechanisms that control central insulin signaling have not been fully elucidated. Previously, we showed that LMO4 is highly expressed in hypothalamic nuclei that regulate glucose homeostasis. Here, we determined how loss of LMO4 in the hypothalamus would affect central insulin signaling and glucose homeostasis. In transgenic mice that have LMO4 in ablated in glutamatergic neurons, we found that insulin signaling is impaired in the hypothalamus as well as in peripheral tissues (liver and skeletal muscle). Impaired glucose homeostasis was associated with a markedly elevation in hypothalamic protein tyrosine phosphatase 1B (PTP1B) activity. PTP1B is a key phosphatase that terminates insulin signaling by dephosphorylating its receptor and downstream signaling molecules. Importantly, we found that administration of a selective PTP1B inhibitor Trodusquemine to the hypothalamus restored central insulin signaling and improved the response of peripheral tissues to insulin in these LMO4-deficient mice. Thus, our study reveals an essential requirement for LMO4 to modulate central insulin signaling.

1. Introduction

Hypothalamic insulin signaling regulates key metabolic functions, including glucose homeostasis and energy expenditure [1]. Whereas peripheral insulin signaling has been extensively characterized, the mechanisms that regulate the central effects of insulin are less well studied. Hypothalamic insulin signaling controls glucose homeostasis by suppressing glucose production from the liver [2,3] and by increasing glucose uptake by muscle [4]. Central insulin infusion increases parasympathetic outflow to the liver and surgical resection of the hepatic branch of the vagus nerve blocks the effects of central insulin and reduces the effects of systemic insulin on hepatic glucose production [3]. Ablation of insulin receptor in the hypothalamus is sufficient to block central insulin-mediated suppression of hepatic gluconeogenesis [2]. Central insulin infusion also increases sympathetic outflow to peripheral tissues, including skeletal muscle and brown adipose tissue, to increase their response to peripheral insulin signaling [4]. Whole-body insulin receptor knockdown results in more pronounced hyperinsulinemia and hyperglycemia than peripheral tissue knockdown [5].

In obesity, central sensitivity to insulin is reduced, which impedes the effectiveness of central insulin to control metabolic homeostasis contributing to peripheral insulin resistance and type II diabetes. Insulin receptor activation triggers a cascade of phosphorylation events that mediate its physiological functions [1]. Protein Tyrosine Phosphatase 1B (PTP1B) is a key phosphatase that terminates these intracellular signaling pathways by dephosphorylating phosphotyrosyl residues on insulin receptor and its substrates [1,6]. Elevated PTP1B activity is a major mechanism contributing to insulin resistance. High fat diet elevates hypothalamic PTP1B activity [1] whereas reduction of hypothalamic PTP1B improves insulin resistance in diet-induced obese rats [7].

Hypothalamic insulin resistance results in hepatic insulin resistance, lipid accumulation, and visceral obesity [1]. Mice with brain-specific insulin receptor (IR) knockout are not only defective in hypothalamic insulin signaling but also display hepatic insulin resistance [8]. Significantly, restoration of liver insulin signaling in IR knockout mice failed to normalize insulin action to suppress

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hepatic glucose production [9]. Moreover, mice with brain-specific PTP1B knockout have improved peripheral insulin sensitivity even when fed a high fat diet [10]. Together, these studies point to the importance of hypothalamic insulin signaling to maintain peripheral insulin signaling and glucose homeostasis.

Recently, we discovered that the small LIM domain only protein LMO4 is an endogenous inhibitor of PTP1B [11]. We found that LMO4 is sensitive to metabolic and other stresses that regulate its inhibition of PTP1B [11,12]. These findings stemmed from the observation that mice with glutamatergic neuron-specific ablation of LMO4, CaMK2αCre/LMO4flox (LMO4KO) mice, develop early-onset adiposity, progressing to hyperphagia and obesity. The metabolic phenotype was attributed to reduced sympathetic outflow to adipose tissue [13]. Since LMO4KO mice have elevated central PTP1B activity [11] and PTP1B is known to affect central insulin signaling [14], we hypothesized that these mice have hypothalamic insulin resistance that could contribute to peripheral insulin resistance.

Here, we report that LMO4KO mice have central insulin resistance due to elevated PTP1B activity at the hypothalamus. Further, we show that central PTP1B inhibition restores both central (hypothalamic) and peripheral (liver and skeletal muscle) insulin sensitivity in LMO4KO mice. Thus, LMO4 plays a key role to maintain hypothalamic insulin signaling and control glucose homeostasis.

2. Materials and methods

2.1. Animal

LMO4KO (CaMK2αCre/LMO4flox) mice were bred in a CD1 background and fed regular chow as described previously [11]. Male mice were used for all experiments.

2.2. Intracerebroventricular (icv) Trodusquemine and insulin injection

Two-month-old mice were anaesthetized and placed in a stereotactic apparatus to install a 21-gauge stainless-steel guide cannula (Plastics One) at the midline, 1.7 mm posterior to the bregma and 5.6 mm ventral to the dura. Mice received icv injection of Trodusquemine (30 g/kg in 1 μl) [15] or vehicle (saline) right after cannulation. On day 5, mice were fasted for 6 h and 1 μl of insulin (1 mU/kg) or saline was injected to conscious mice via the cannula. 10 min after insulin injection, mice were killed and tissues were isolated within 2 min and snap frozen till use.

2.3. Acute hypothalamic slice wedges and insulin response

Three 400 μm coronal vibratome sections from bregma −1.1 to −2.3 mm were obtained per mouse, and square hypothalamic wedges were removed, incubated in artificial cerebrospinal fluid [16] at room temperature for 20 min and at 37 °C for 1 h, followed by insulin (100 nM) treatment for various times [11]. For each time point, six hypothalamic wedges were pooled from two mice (containing all three hypothalamic wedges per mouse to avoid rostrocaudal variation).

2.4. Cell culture and transfections

F11 neuronal cells were transfected using Lipofectamine2000 (Invitrogen) with LMO4 or empty expression vector pcDNA3, LMO4-specific shRNA or control scrambled shRNA expression vectors [17].

2.5. PTP1B phosphatase activity assay

PTP1B phosphatase activity was measured in extracts from hypothalamic wedges or F11 cells using the PhosphoSeek PTP1B Assay Kit (BioVision) with proper positive and negative controls [11].

2.6. Immunoblot analyses

Tissue and cells lysates were harvested and analyzed subjected to SDS–PAGE immunoblot analysis as described [11,18]. The same blots were subsequently re-probed with corresponding total antibodies to detect the total amount of these proteins. Antibodies were obtained from Cell Signaling Inc (pIR1β (pY1345), IRS1 (pY895), Akt (pS473), GSK3β (pS9), IR1β, IRS1, Akt, GSK3β, β-actin, GAPDH), Santa Cruz (Glut4) and Millipore (Na+/K+ ATPase).

2.7. Statistical analyses

All results are expressed as mean ± SEM and analyzed with SPSS Software using a two-tailed unpaired Student's t test or ANOVA. $p < 0.05$ was considered significant.

3. Results

3.1. LMO4 is required for insulin signaling in the hypothalamus

Previously, we showed that LMO4, by inhibiting PTP1B, is required for the hypothalamic response to leptin [11]. Since PTP1B also dephosphorylates insulin receptor and signaling components, we asked whether LMO4 affects insulin signaling in neuronal cells and in the hypothalamus. Using F11 neuronal cells, LMO4 expression was knocked down by transient transfection using an LMO4 shRNA [18]. As a control, cells were transfected with a scrambled shRNA. We found that protein extracts from cells stimulated with insulin (100 nM) for 5 min showed increased levels of tyrosine-phosphorylated insulin-receptor substrate-1 (pIRS-1) in control shRNA-transfected cells but not in cells where LMO4 was knocked down (Fig. 1A). Similarly, activation of Akt (pAkt) was blunted with LMO4 knockdown. Pretreating cells with the phosphatase inhibitor sodium orthovanadate (1 mM) 30 min prior to insulin stimulation restored pIRS1 in cells where LMO4 was knocked down (Fig. 1B). Conversely, over-expression of LMO4 increased pIRS1 levels in response to insulin stimulation and these levels were further elevated by sodium orthovanadate (Fig. 1C). These results show that LMO4 regulates the response to insulin in neuronal cells.

To test whether LMO4 ablation also affects the response of the hypothalamus to insulin stimulation, brain slices through the hypothalamus were isolated from LMO4KO and littermate control (WT) mice and stimulated with 100 nM insulin for 10 and 30 min. Proteins were extracted from the hypothalamus pooled from 3 brain slices at each time point. Insulin induced a rapid and transient phosphorylation of the insulin receptor (pIR-β) in WT but not in LMO4KO hypothalamus (Fig. 1D). Similarly, pAkt levels were elevated upon insulin stimulation in WT hypothalamus. In contrast, LMO4KO hypothalamus had higher basal levels of pAkt that declined with insulin stimulation (Fig. 1D). These results indicate that LMO4 is required for central insulin signaling in the hypothalamus.

3.2. PTP1B inhibitor Trodusquemine restores insulin signaling in LMO4KO hypothalamus

Since LMO4 is an endogenous inhibitor of protein tyrosine phosphatase 1B (PTP1B) [11] and PTP1B dephosphorylates the

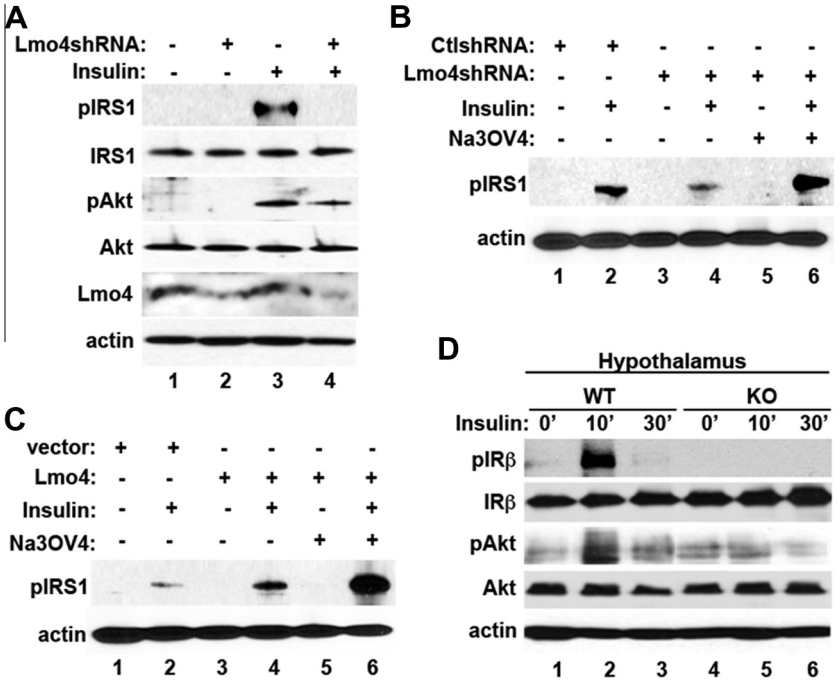

Fig. 1. LMO4 is required for insulin signaling in neurons. (A) LMO4 knockdown by shRNA in F11 neuronal cells impairs their response to insulin (100 nM, 10 min), as revealed by reduced phosphorylated insulin receptor substrate 1 (pIRS1) and pAkt in immunoblots. Insulin-induced phosphorylation of IRS1 (pIRS1) was blunted by LMO4 knockdown (B) and was augmented by overexpression of LMO4 (C). Treatment with phosphatase inhibitor sodium orthovanadate (Na_3OV_4) 30 min prior to insulin stimulation restored or increased tyrosine phosphorylation of IRS-1 with LMO4 knockdown or overexpression, respectively. (D) Hypothalamic wedges from LMO4KO mice showed an impaired response to insulin over a 30 min time course of *in vitro* treatment compared to littermate controls (WT), as revealed by reduced phosphorylated insulin receptor beta (pIR-β) and pAkt. β-actin was used as a loading control for total protein.

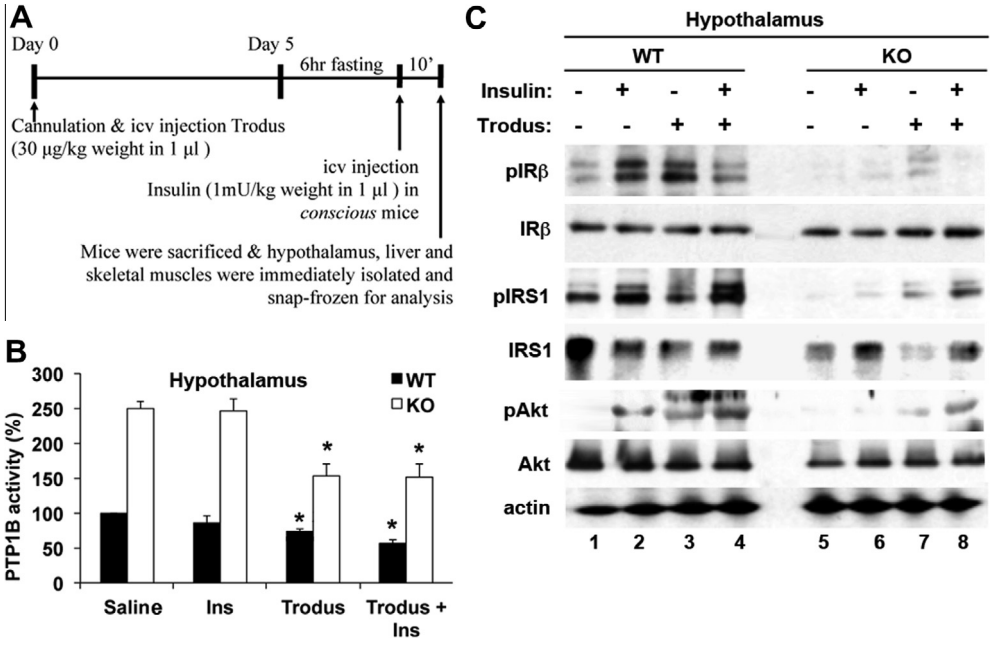

Fig. 2. Central Trodusquemine administration restored insulin signaling in the hypothalamus. (A) Schematic representation of *in vivo* experimental manipulation. (B) Elevated hypothalamic PTP1B activity in LMO4KO hypothalamus was blocked by central Trodusquemine administration. Acute insulin (Ins) treatment did not affect PTP1B activity. PTP1B activity was normalized to saline treated WT mice and expressed as % mean ± SEM. $^*p < 0.05$. $n = 3$ mice per group. (C) Central Trodusquemine administration restored phosphorylation of IR-β, IRS-1, Akt and GSK3-β in response to insulin in LMO4KO hypothalamus and potentiated insulin's effect on these signaling markers. β-actin, loading control for total protein.

insulin receptor and its downstream signaling molecule IRS1 [19], we asked whether selective inhibition of PTP1B would restore insulin signaling in the hypothalamus of LMO4KO mice. Intracerebroventricular (icv) cannulae were implanted into mice to inject the PTP1B-selective inhibitor Trodusquemine (30 µg/kg in 1 µl) or saline into the third ventricle 5 days prior to icv insulin injection (1 mU in 1 µl). Trodusquemine is long acting *in vivo*, with a sustained inhibition of PTP1B at least 5 days after a single icv injection

[11]. Mice were fasted for 6 h prior to insulin injection and hypothalamus was harvested 10 min after insulin treatment, according to the scheme shown in Fig. 2A.

PTP1B activity was markedly elevated in hypothalamic extracts from LMO4KO mice, and Trodusquemine lowered PTP1B activity in both LMO4KO and WT mice, as we reported previously [11] (Fig. 2B). Of note, previously we reported 3.5-fold higher activity without fasting [11], here we observed 2.5-fold higher PTP1B activity in LMO4KO hypothalamus after 6 h of fasting. Ten minutes after icv injection of insulin, little effect on PTP1B activity was observed in either WT or LMO4KO hypothalamus.

Immunoblot analysis showed that insulin increased levels of pIRβ in WT but not LMO4KO hypothalamus (Fig. 2C, compare lanes 2–1 and 6–5). Similarly, phosphorylation of IRS1 and Akt, was detected in WT but not KO hypothalamus. To our knowledge, PTP1B is the principal tyrosine phosphatase that targets IRS1 [19]. IRS1 activates PI3K that phosphorylates Akt [20]. Consistent with these notions, insulin signaling deficits in LMO4KO hypothalamus were rescued by icv injection of Trodusquemine (Fig. 2C, compare lanes 8–6). Interestingly, Trodusquemine treatment alone was sufficient to increase pAkt in both WT and LMOKO hypothalamus (Fig. 2C, lanes 3–7), likely reflecting an increase in the sensitivity to endogenous insulin levels due to lowered PTP1B activity. In summary, insulin signaling is defective in the LMO4KO hypothalamus and can be rescued by the PTP1B inhibitor Trodusquemine as evidenced by increased pIRS1 and pAkt levels in response to insulin.

3.3. Central Trodusquemine administration ameliorates peripheral insulin resistance in LMO4KO mice

Hypothalamic insulin signaling is important to maintain glucose homeostasis. Whole-body insulin receptor ablation results in more pronounced hyperinsulinemia and hyperglycemia than peripheral tissue ablation [5]. Knockdown of insulin receptor in the hypothalamus blocks central insulin-mediated suppression of hepatic glucose production [2]. Consistent with impaired central insulin signaling, circulating insulin levels are elevated in LMOKO mice by the age of 2 months and these mice become insulin resistant as shown by insulin and glucose tolerance tests, as we reported previously [11]. As expected, PTP1B activity in the liver, an indicator of insulin resistance [21], was higher in LMO4KO mice compared to littermate controls (Fig. 3A). Central Trodusquemine treatment lowered PTP1B activity in the liver of WT and LMO4KO mice (Fig. 3A).

Central insulin infusion activates parasympathetic outflow via the vagus nerve to suppress hepatic glucose production [3,22]. Glycogen synthase kinase 3-β (GSK3β) is the key regulator controlling the expression of gluconeogenic genes and its activity is suppressed by Akt-dependent phosphorylation in response to insulin [23]. In line with these studies, a rapid phosphorylation (i.e. inactivation) of GSK3β in the liver was observed in WT mice 10 min after central infusion of insulin. A correlated increase in its upstream kinase and signaling regulators, pAkt, pIRS1 and pIR1β, were also observed (Fig. 3B, lane 2). In contrast, LMO4KO liver fails to respond to central insulin stimulation and pAkt and pGSK3β were not induced (Fig. 3B, lane 6), consistent with an impaired response to central insulin signaling in the hypothalamus of these mice (Fig. 2C).

Since central Trodusquemine administration restored the central response to insulin in LMO4KO hypothalamus (Fig. 2C), we asked whether it would also restore the insulin response of peripheral tissues. Indeed, Trodusquemine increased pIRS1, pAkt and pGSK3β levels in LMO4KO liver (Fig. 3B, compare lanes 8–6). These results show that the hepatic response to insulin can be restored by central administration of Trodusquemine.

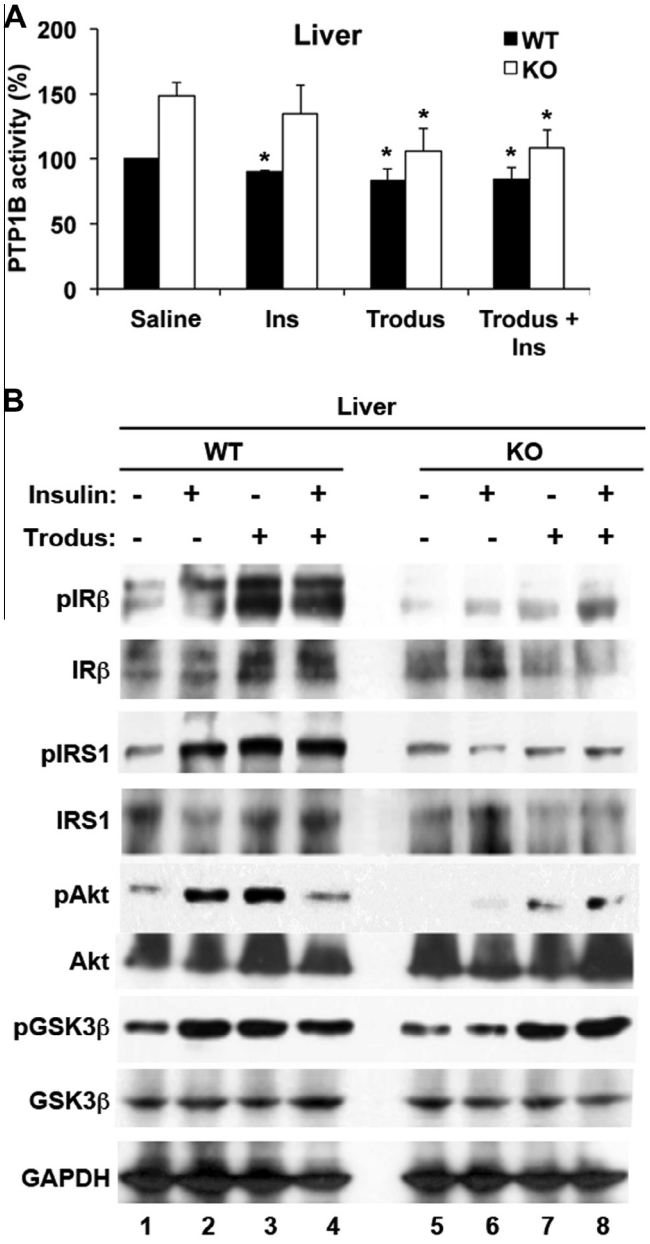

Fig. 3. Central Trodusquemine administration restored the response to insulin in liver. (A) Elevated PTP1B activity in LMO4KO liver was lowered by central Trodusquemine administration. $^{*}p < 0.05$. $n = 3$ mice per group. (B) Central Trodusquemine administration restored phosphorylation of IR-β, IRS-1, Akt and GSK3-β in LMO4KO skeletal muscle in response to central insulin infusion. GAPDH, loading control for total protein.

Central insulin infusion also increases sympathetic outflow to peripheral tissues, including skeletal muscle and brown adipose tissue, to increase IRS1 activation and peripheral insulin signaling [4]. Indeed, we observed elevated pIRS1 and pAKT levels in WT skeletal muscle after central insulin infusion, associated with incorporation of the glucose transporter Glut4 into the cell membrane to increase muscle glucose uptake (Fig. 4B, lane 2). In contrast, LMO4KO skeletal muscle did not respond to central insulin infusion (Fig. 4B, lane 6), consistent with central insulin resistance (Fig. 1D and Fig. 2C).

In contrast to the observed reduction in PTP1B activity in the liver of WT and LMO4KO mice (Fig. 3A), central Trodusquemine lowered PTP1B activity in WT but not in LMO4KO skeletal muscle (Fig. 4A). Nonetheless, central Trodusquemine infusion restored

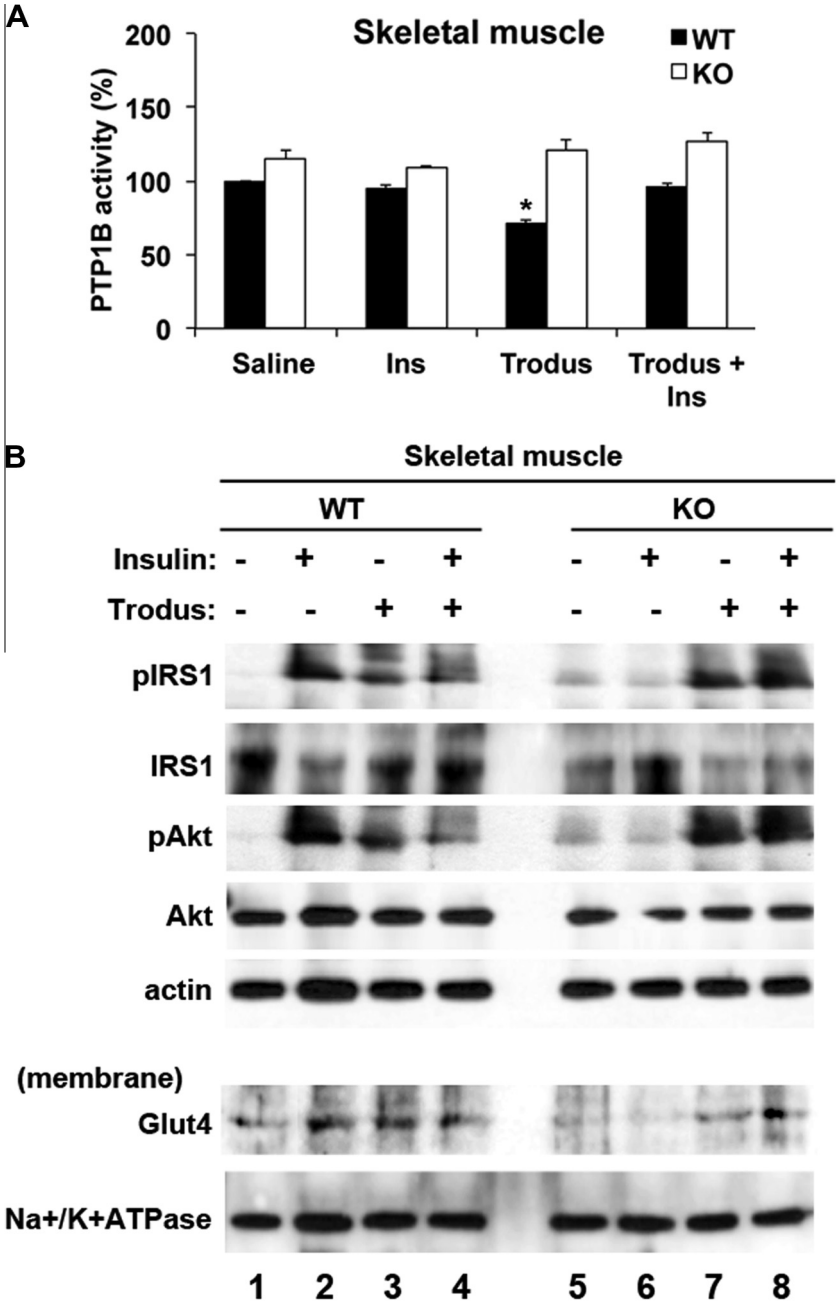

Fig. 4. Central Trodusquemine administration restored the response to insulin in skeletal muscle. (A) Central Trodusquemine treatment did not alter PTP1B activity in the LMO4KO skeletal muscle. $^{*}p < 0.05$. $n = 3$ mice per group. (B) Central Trodusquemine administration restored phosphorylation of IRS-1 and Akt in LMO4KO skeletal muscle in response to central insulin infusion. Glut4 translocation to the membrane of skeletal muscle in response to central insulin infusion was restored by central Trodusquemine administration in LMO4KO mice. Loading control for the membrane fraction (Na+/K+ ATPase) and for total protein (β-actin).

pIRS1 and pAkt levels in LMO4KO skeletal muscle (Fig. 4B, lane 8). Consistent with the restoration of central insulin signaling by Trodusquemine, it also restored Glut4 membrane incorporation in skeletal muscle of LMO4KO mice in response to central insulin infusion (Fig. 4B, lane 8). Of note, Trodusquemine also elevated pIRS1, pAkt and membrane Glut4 levels even without exogenous insulin stimulation in both WT and LMO4KO (Fig. 4B, lanes 3 and 6). Taken together, our results show that central insulin signaling is impaired in the hypothalamus of LMO4KO mice and that inhibition of PTP1B by Trodusquemine restores central insulin signaling and thereby ameliorates peripheral insulin sensitivity in these mice.

4. Discussion

Our study has demonstrated that LMO4 is required for central insulin signaling. We showed that LMO4KO mice have an impaired response to central insulin action that can be restored by inhibiting central PTP1B activity with Trodusquemine. Central insulin action is important to control glucose homeostasis by suppressing hepatic gluconeogenesis and by promoting glucose uptake in skeletal muscle. As a consequence of deficient central insulin signaling in LMO4KO mice, insulin signaling controlling glucose homeostasis in peripheral tissues, including the liver and skeletal muscle, is also compromised.

PTP1B activity was highly elevated (2.5-fold) in the hypothalamus of LMO4KO mice due to ablation of its endogenous inhibitor LMO4 [11] in hypothalamic glutamatergic neurons. PTP1B was elevated only 1.5-fold in the liver and 1.2-fold in skeletal muscle. The modest elevation of PTP1B activity in the liver and skeletal muscle of LMO4KO mice is likely an indirect consequence of insulin resistance present at 2 months of age [11].

Central administration of Trodusquemine lowers PTP1B activity in the hypothalamus of both WT and LMO4KO mice. Even without additional insulin infusion, Trodusquemine alone activates the signaling pathway, suggesting that it augments the sensitivity to existing insulin levels by lowering PTP1B activity. That central Trodusquemine also lowers PTP1B activity in the liver was surprising. Although Trodusquemine can cross the blood brain barrier [15,24], the fact that PTP1B activity was not lower in LMO4KO skeletal muscle argues against a direct effect of Trodusquemine to lower liver PTP1B activity. In addition, the dose administered by icv injection is 100–300 times lower than the dose normally used systemically [15,24]. One likely explanation to account for the lowered PTP1B activity in the liver is that central Trodusquemine activates parasympathetic outflow to the liver and lowers PTP1B activity. Activation of acetylcholine receptors upon parasympathetic stimulation is known to activate Akt [25], which would phosphorylate and inhibit PTP1B [26]. As a result, this would lower PTP1B activity in the liver, but not in skeletal muscle that does not receive parasympathetic innervation.

Despite the lack of reduced PTP1B activity in LMO4KO skeletal muscle, central Trodusquemine did restore insulin signaling in LMO4KO skeletal muscle. This result likely reflects the central action of Trodusquemine to stimulate sympathetic outflow [11] to enhance insulin sensitivity of skeletal muscle, since activation of beta-adrenergic receptors has been reported to enhance Akt phosphorylation and the response to insulin [27]. This mechanism would also contribute to increase insulin sensitivity in the liver.

The present study has focused on one aspect of LMO4 function, namely its inhibition of PTP1B. LMO4 is a small protein bearing 2 LIM protein interaction domains that interact with cytoplasmic and membrane associated receptors and phosphatases, including PTP1B [11,18,28,29]. In addition, LMO4 is present in the nucleus and interacts with several transcription factors and regulates their activities [30,31]. Thus, other mechanisms could also be disrupted by LMO4 ablation and contribute to impaired insulin signaling in LMO4KO mice.

To appreciate how LMO4 regulates insulin action within the hypothalamus, it is important to understand how insulin activates neural circuits and autonomic outflow. Insulin hyperpolarizes SF1 neurons located in the ventromedial hypothalamus (VMH), which provide glutamatergic input on pro-opiomelanocortin (POMC) neurons at the arcuate nucleus (ARC) [32]. POMC neurons project to the paraventricular hypothalamic nucleus (PVN) and activate sympathetic outflow to peripheral organs including skeletal muscle, liver and pancreas [33]. LMO4 is highly expressed in the VMH and PVN but not in the ARC [13]. Mice with ablation of LMO4 in the glutamatergic neurons have reduced sympathetic outflow associated with impaired insulin signaling (present study) and leptin signaling [11,13] at the hypothalamus. Thus, the disruption of insulin signaling in LMO4KO hypothalamic circuits could occur at the VMH and/or PVN.

Our recent study reported that selective ablation of LMO4 in Sim1 neurons of the PVN (Sim1Cre/LMO4flox mice) reduces neuronal excitability and causes severe hyperphagia, obesity and insulin resistance [34]. Importantly, and in contrast to LMO4KO mice (CamK2αCre/LMO4flox) where paired feeding does not restore insulin signaling [11], insulin resistance was prevented by paired feeding of Sim1Cre/LMO4flox mice [34]. These two studies suggest that either LMO4 in the PVN is not required for central insulin signaling or that insulin does not act directly on Sim1 neurons of the PVN to activate sympathetic outflow to the periphery. Interestingly, insulin injected locally into the arcuate nucleus increased sympathetic activity, but had no effect when injected into the PVN [35]. Apart from the PVN, the arcuate nucleus also projects to other brain regions that regulate the sympathetic outflow, including the dorsomedial hypothalamus, midbrain periaqueductal grey, rostral ventrolateral medulla and the nucleus of the solitary tract [36]. Thus, neurons of the PVN may relay the signals of central insulin action at the VMH and ARC rather than respond directly to insulin. Together, our studies suggest that LMO4 in the VMH bears the onus of maintaining central insulin action. Future studies combining electrophysiology with molecular genetic techniques using mice with VMH-selective ablation of LMO4 (SF1Cre/LMO4flox) will be needed to address this question.

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