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Modulation of hypothalamic PTP1B in the TNF-α-induced insulin and leptin resistance

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ABSTRACT

We have associated functional and molecular studies of insulin and leptin to investigate the effect of TNF-α on central insulin and leptin signaling in rats pre-treated with PTP1B-ASO. The icv infusion of TNF-α-induced an increase in PTP1B protein expression and activity, and attenuated insulin and leptin sensitivity and signaling in the hypothalamus. However, TNF-α was able to completely blunt the leptin and insulin effect in rats treated with PTP1B-ASO, suggesting that TNF-α does not require PTP1B to fully attenuate the leptin and insulin effects. In addition, our data also show that other mechanisms of insulin and leptin resistance are activated in the hypothalamus by TNF-α.

1. Introduction

Obesity is accompanied by resistance to insulin and leptin, key hormones regulating glucose homeostasis and body weight [1], but the molecular mechanisms that account for these hormonal resistances are not completely known. The protein tyrosine phosphatase – PTP1B is one of the major negative regulators of insulin and leptin signaling, acting to dephosphorylate the insulin receptor and the leptin receptor-associated Janus kinase 2 [2,3]. In previous studies, we and others demonstrated that a selective decrease in hypothalamic PTP1B protein resulted in decreased food intake, reduced body weight, reduced adiposity after high-fat feeding and improved insulin and leptin action and signaling [4,5]. Other studies demonstrated that the PTP1B deficiency enhances insulin signaling, in skeletal muscle and liver, reduces adiposity and protected from diet-induced obesity [5–10].

In most studies in obese animals, in different tissues, an increase in phosphotyrosine phosphatase activity is well characterized, and specifically an increase in PTP1B expression and activity. It is important to mention that there is a clear correlation between the increase in PTP1B tissue level with enzyme activity [11–13,4], suggesting that the regulation of PTP1B protein expression as a major mechanism mediating increased PTP1B activity. However the mechanisms that influence PTP1B level/activity in obesity are not completely known. In the past 10 years it became evident that in obesity and type 2 diabetes there is a subclinical inflammatory process, which has an important role in the insulin resistance in these situations. Elevated inflammatory cytokines such as tumor necrosis factor α (TNF-α), IL1β, and IL6 are seen in obesity and may play a direct role in development of obesity-associated insulin resistance [14–16].

In peripheral tissues TNF-α impairs insulin signal transduction through the activation of serine kinases, such as JNK and IKKβ [17–19]. Moreover, a recent study has shown that adipose tissue inflammation and the pro-inflammatory cytokine, TNF-α, can regulate PTP1B expression in vivo [20]. Since TNF-α may, under different circumstances, antagonize the effects of insulin and leptin in the hypothalamus we decided to evaluate the effects of TNF-α on the regulation of PTP1B in the hypothalamus, and also whether this cytokine is able to antagonize insulin and leptin action and signaling, after a reduction in PTP1B expression in this tissue. Knowledge of the regulation of PTP1B in the central nervous system may show metabolic and functional importance in the regulation of insulin and leptin signaling in the brain, providing substrate for new therapeutic approaches.

Abbreviations: IR, insulin receptor; IRS, insulin receptor substrate; icv, intracerebroventricular infusion; PTP1B, protein tyrosine phosphatase 1B

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2. Materials and methods

2.1. Antibodies, chemicals, buffers and oligonucleotides

Antibodies anti-IR, IRS-1, Jak-2, Stat3, anti-phospho-JNK (phosphorylated at Thr^{183} and Tyr^{185}), anti-phospho-IRS-1(Ser^{307}), anti-phospho-IKKβ (phosphorylated at Ser^{181}) and anti-phosphotyrosine were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphoserine antibody was purchased from Zymed Laboratories Inc., San Francisco, CA. Anti-phospho-Akt (rabbit polyclonal, recognizing Akt phosphorylated at Ser^{473}) was purchased from Upstate Biotechnology (Charlottesville, VA, USA) Anti-PTP1B (AB-1 mouse polyclonal) was purchased from Calbiochem (La Jolla, CA). pp60src C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL; where pY represents phosphotyrosine and Biomol Green reagent were purchased from Biomol (Plymouth Meeting, PA). Reagents for SDS/PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA). Tris [hydroxymethyl] amino-methane (Tris), Hepes, phenylmethysulfonyl fluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-I00, glycerol, Tween 20, bovine serum albumin (BSA, fraction V) and angiotensin II were from Sigma Chemical CO. (St. Louis, MO, USA). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), nitrocellulose membranes Human recombinant insulin was from Eli Lilly Co. (Indianapolis, IN, USA). Leptin was from Calbiochem (San Diego, CA, USA). Ketamine hydrochloride was from Fort Dogde Laboratories Inc. (Fort Dodge, IA, USA). Sodium thiopental was from Abbout Laboratories (North Chicago, Illinois, USA). Antisense phosphorthioate oligonucleotides specific for PTP1B (5′-CCA ACA GCA CTT T-3′) was produced by Invitrogen Corp. (Carlsbad, CA). Mouse recombinant TNF-α was from Calbiochem (Darmstadt, Germany).

3. Experimental animals

Eight-week old male Wistar rats (Rattus norvegicus) from the University of Campinas Central Animal Breeding Center were used in the experiments. The rats were allowed access to standard rodent chow and water (ad libitum). Food was withdrawn 12 h before the experiments. All experiments were conducted according to the principles and procedures described by the NIH Guidelines for the Care and Use of Section 3 and were approved by the State University of Campinas Ethical Committee. In all experiments, the rats were cannulated intracerebroventricularly (icv) and submitted to treatment with PTP1B-ASO (accompanied or not by saline, insulin, leptin, TNF-α or combinations of TNF-α with insulin or leptin, according to the protocols described below.

3.1. Intracebroventricular cannulation

All rats were stereotaxically instrumented using a Stoelning stereotaxic apparatus, according to a previously described method [21]. Cannula efficiency was tested 1 week after cannulation by the evaluation of the drinking response elicited by icv angiotensin II [22].

3.2. Phosphorthioate-modified oligonucleotide treatment

The sequences of antisense phosphorothioate oligonucleotides, specific for PTP1B 5′-CCA ACA GCA CTT T-3′), were selected among three unrelated pairs of oligonucleotides on the basis of their ability to block PTP1B protein expression, as evaluated by immunoblotting total protein extracts of hypothalamus using specific anti-PTP1B antibody.

The antisense oligonucleotide sequences were submitted to BLAST analyses (www.ncbi.nlm.nih.gov) and matched only for the Rattus norvegicus PTP1B coding sequence. Rats were cannulated, housed in individual cages and treated with saline or PTP1B antisense oligonucleotide (PTP1B-ASO). PTP1B-ASO was diluted in TE buffer (10 mM Tris–HCl, 1 mM EDTA) and treatment was achieved by icv infusions, twice a day (8:00 h/18:00 h) with a total vol of 2.0 μl per dose (4.0 nmol/μl) for 4 days.

3.3. Protocol for food ingestion determination

Rats cannulated icv were treated with PTP1B-ASO or saline twice a day (8:00 h/18:00 h) with a total vol of 2.0 μl per dose (4.0 nmol/μl) for 4 days. At the end of the fourth day, rats were treated icv with insulin (2.0 μl, 10^{-6} M), leptin (2.0 μl, 10^{-6} M), TNF-α (2.0 μl, 10^{-12} M), saline or with combinations of TNF-α (2.0 μl, 10^{-12} M) with insulin (2.0 μl, 10^{-6} M) or leptin (2.0 μl, 10^{-6} M). Food ingestion was determined over the next 12 h.

3.4. Tissue extraction, immunoblotting and immunoprecipitation

Rats pre-treated with PTP1B-ASO or saline for 4 days were anesthetized with sodium thiopental and, as soon as anesthesia was assured by the loss of pedal and corneal reflexes, the rats were subjected to craniotomy 15 min after the initial insulin (2.0 μl, 10^{-6} M), leptin (2.0 μl, 10^{-6} M) or saline (2.0 μl) and 4 h after the initial TNF-α infusion. The hypothalami were obtained and homogenized in freshly prepared ice-cold buffer (1% Triton X-100, 100 mM Tris, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.01 mg aprotinin/ml). Insoluble material was removed by centrifugation (15 000×*g* rpm) for 40 min at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye binding method [23]. Aliquots of the resulting supernatants containing 2.0 mg of total protein were used for immunoprecipitation with specific antibodies, at 4 °C overnight, followed by addition of protein A-Sepharose 6 MB for 2 h. The pellets were washed three times in ice-cold buffer (0.5% Triton X-100, 100 mM Tris, pH 7.4, 10 mM EDTA, and 2 mM sodium vanadate) and then resuspended in Laemmli sample buffer [24] and boiled for 5 min, after which they were subjected to SDS–PAGE in a miniature slab gel apparatus (Mini-Protean). For total extracts, 0.2 mg of protein was separated by SDS–PAGE. Electrotransfer of proteins from the gel to nitrocellulose was performed for 120 min at 120 V in a Bio-Rad Mini-Protean transfer apparatus. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter for 2 h in blocking buffer (5% non-fat dry milk, 10 mM Tris, 150 mM NaCl, 0.02% Tween 20) [25]. The nitrocellulose blot was incubated with specific antibodies overnight at 4 °C and then incubated with ^{125}I-labeled protein A. Results were visualized by autoradiography with pre-flashed Kodak XAR film. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software, ScionCorp).

3.5. Protein tyrosine phosphatase activity assay

The in vitro PTP1B activity assay was conducted based on a protocol previously described by Taghibiglou et al. [13]. Hypothalami were removed and homogenized in solubilization buffer containing 1% Triton X-100, 20 mM, Tris (pH 7.6), 5 mM EDTA, 2 mM PMSF and 0.1 mg aprotinin/mL, 1 mM EGTA and 130 mM NaCl. Lysates were centrifuged (15 000×*g* rpm, 40 min, 4 °C) and the supernatants were collected for immunoprecipitation with anti-PTP1B antibody, as described previously. Immunoprecipitate was washed in PTP assay buffer (100 mM HEPES (pH 7.6), 2 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 0,5 mg/mL bovine serum

albumin). The pp60c-src C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL) was added to a final concentration of 200 μM in a total reaction volume of 60 μl in a PTP assay buffer for immunoprecipitation. The activity of total extracts (125 μg) was measured in the same manner in a total reaction volume of 60 μl in a PTP assay buffer, adding the peptide to a final concentration 200 μM. The reaction was then allowed to proceed for 1 h at 30 °C. At the end of the reaction, 40 μl aliquots were placed into 96-well plates, 100 μl of Biomol Green reagent (Biomol) was added, and absorbance was measured at 660 nm.

3.6. Data presentation and statistical analysis

Data were expressed as means ± S.E.M. accompanied by the indicated number of independent experiments. For statistical analysis, the groups were compared using a two way ANOVA with the Bonferroni test for post hoc comparisons. The level of significance adopted was $P < 0.05$.

4. Results

4.1. Activation of PTP1B by TNF-α in the hypothalamus

TNF-α inhibits insulin action in vitro and in vivo by altering expression or activity of multiple proteins in the insulin signaling pathway in cells [15,26,27]. The up-regulation of PTP1B expression was recently found in cells treated with TNF-α [28]. To evaluate the ability of TNF-α to modulate PTP1B expression and activity in hypothalamus, icv cannulated rats were treated with a single dose of TNF-α (2.0 μl, 10^{-12} M) or saline and the hypothalami were obtained 1, 2 and 4 h after the initial TNF-α infusion. As shown in Fig. 1A, the expression of PTP1B increased by 50% ($P < 0.05$) at 2 h after treatment with TNF-α, and a more marked increase was observed after 4 h (∼148% – $P < 0.05$).

To address the nature of the change in PTP1B that accompanies the increase in expression in response to TNF-α, hypothalami extracts were used to examine the phosphoserine and phosphotyrosine content of PTP1B by immunoblotting with anti-phosphoserine and anti-phosphotyrosine antibodies. Using the peak response at 4 h following TNF-α administration icv, the phosphoserine content of PTP1B increased significantly by 150% ($P < 0.05$) and phosphotyrosine content decreased by about 50%, respectively ($P < 0.05$) (Fig. 1B and C). We next investigated whether the total PTPase and PTPase, related to PTP1B activities, accompanies the increase in the expression of PTP1B in response to TNF-α. The administration of TNF-α-induced increases in the phosphatase activity of PTP1B and in total phosphatase activity of 147% ($P < 0.05$) and 145% ($P < 0.05$), respectively (Fig. 1D and E).

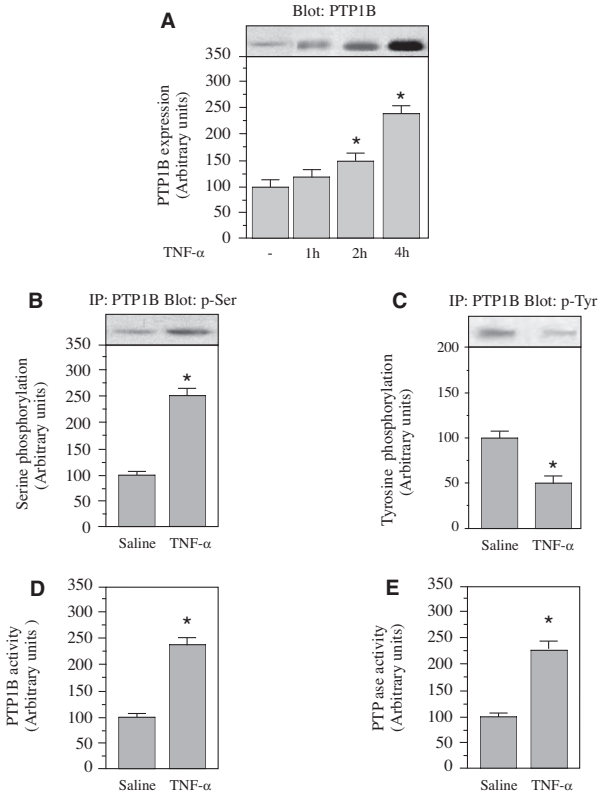

Fig. 1. Activation of PTP1B by TNF-α in hypothalamus. Immunoblotting with anti-PTP1B antibody of whole tissue extracts from hypothalamus of rats treated with TNF-α (10^{-6} M) for 1, 2 and 4 h (A); Immunoprecipitate with anti-PTP1B antibody of whole tissue extracts from hypothalamus of rats treated with TNF-α (10^{-6} M) for 4 h and followed by immunoblotting with antibody anti-phosphoserine (B) or anti-phoshotyrosine (C); Total PTPase activity and PTPase activities in immunoprecipitates were assayed by incubation with the pp60c-src C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL). PTPase activity in PTP1B immunoprecipitates (D), Total PTPase activity in hypothalamus (E); in all experiments, $n = 6$; $P < 0.05$; *, vs. saline; Results are presented as means ± S.E.M.

4.2. Activation of insulin action by hypothalamic reduction of PTP1B in the presence of TNF-α

To investigate whether PTP1B may mediate the effect of TNF-α effect on food ingestion, rats were icv cannulated and, after the recovery period, they were treated for 4 days with PTP1B-ASO or saline, and on the 4th day, both groups were treated with 10^{-12} M TNF-α. The potency of PTP1B-ASO was tested in a dose–response experiment. Two daily doses of 4 nmol PTP1B were sufficient to inhibit PTP1B expression by 70% in control rats (Fig. 2A). TNF-α alone promoted no change in food intake (18.1 ± 1.3 g), when compared with rats treated with saline (22.5 ± 0.3 g). As expected in animals treated with PTP1B-ASO, there was a marked reduction in food intake when compared with saline or TNF-α and, after infusion of TNF-α in these animals the, reduction in food intake was less evident (Fig. 2B), suggesting that this cytokine may be acting through a pathway that does not involve PTP1B.

We next decided to evaluate whether TNF-α may modulate insulin effects after the reduction of PTP1B. For this purpose, rats were icv cannulated and, after the recovery period, they were treated for 4 days with PTP1B-ASO or saline and, on the 4th day, both groups were treated with insulin 10^{-6} M alone or TNF-α, followed by insulin. Control rats consumed 20.1 ± 0.5 g of chow. Fig. 2C shows that the infusion of insulin promoted significant reductions in food intake to 8.3 ± 0.7 g ($P < 0.05$ compared with saline). The administration of TNF-α plus insulin partially reversed the anorexigenic effects of insulin (TNF-α plus insulin 12.4 ± 0.8 g × insulin 8.3 ± 0.7 g $P < 0.05$) . Fig. 2D shows that the treatment with PTP1B-ASO decreased food intake to 10 ± 0.8 g ($P < 0.05$ compared with the non-treated rats). PTP1B-ASO potentiated insulin-induced suppression of food ingestion to 3.8 ± 0.2 g. However, insulin-induced suppression of food intake in PTP1B-ASO, treated animals was partially impaired by TNF-α, suggesting that TNF-α may have other mediators besides PTP1B.

Insulin promoted significant increases in the Tyr-phosphorylation of IR, IRS-1 and increased Ser^{473}-phosphorylation of Akt. The co-injection of TNF-α with insulin attenuated insulin-stimulated IR, IRS-1 tyrosine phosphorylation and Akt serine phosphorylation. We explored whether the deleterious effect of TNF-α may be reversed through the reduction of hypothalamic PTP1B. Rats treated with PTP1B-ASO presented a significant increase in insulin-in-

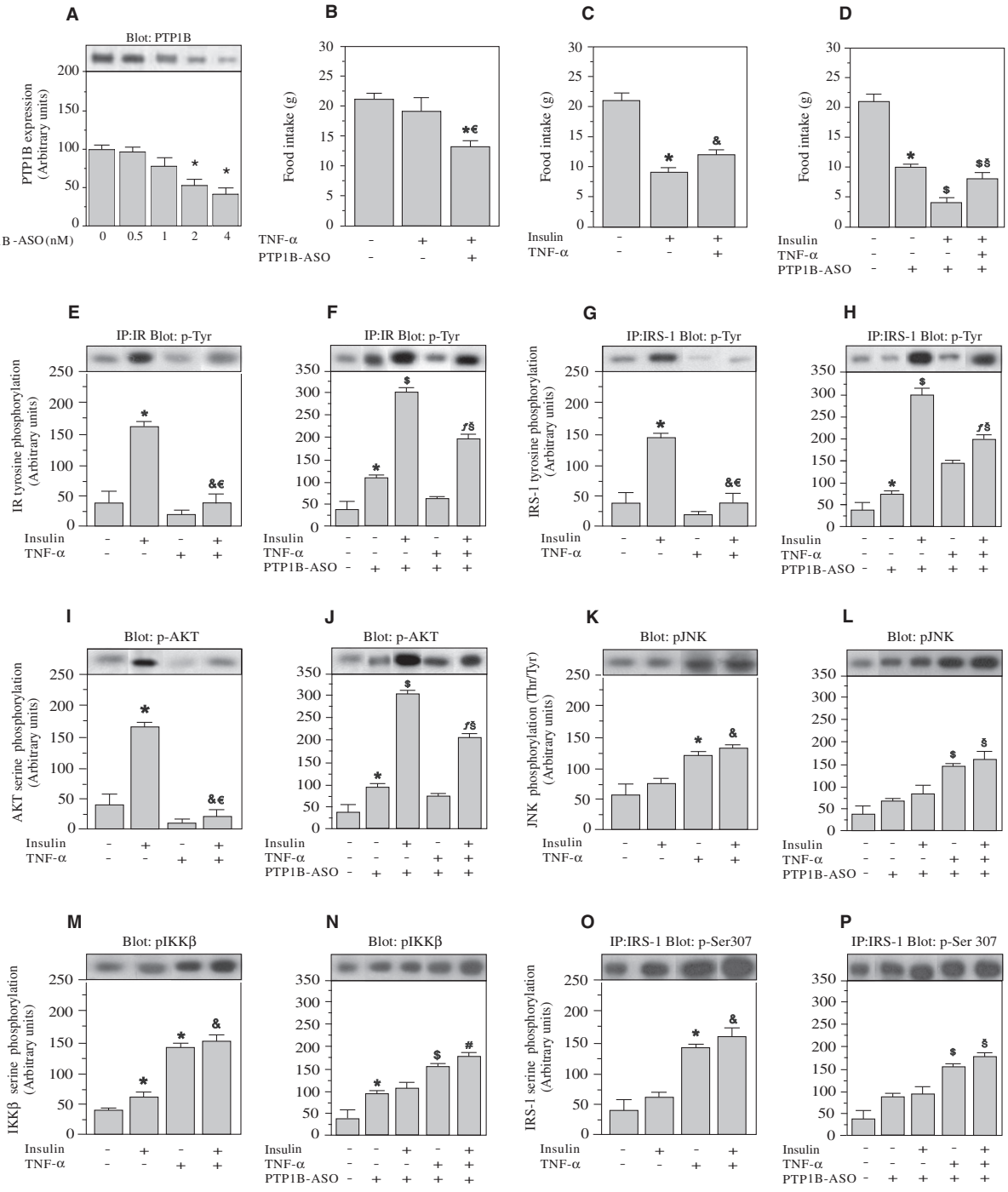

Fig. 2. Activation of insulin action by hypothalamic reduction of PTP1B in the presence of TNF-α. Rats cannulated icv were treated with PTP1B-ASO or saline twice a day (4.0 nmol/μl) for 4 days. The potency of PTP1B-ASO was tested in a dose–response experiment (0.5, 1, 2 and 4 nM) (A). Food ingestion was determined after icv infusion with TNF-α (2.0 μl, 10^{-12} M) in rats pre-treated with PTP1B-ASO or no (B); insulin or TNF-α plus insulin (C); insulin or TNF-α plus insulin in rats pre-treated with PTP1B-ASO (D). To evaluate the insulin signaling, rats pre-treated with PTP1B-ASO or saline for 4 days were anesthetized and subjected to craniotomy 15 min after the initial insulin (10–6 M) or saline icv infusion and 4 h after the initial TNF-α icv infusion; $n = 15$. The hypothalami were obtained for protein extract preparation. The samples were immunoprecipitated (IP) with antibodies anti-IR (E, F) and IRS-1 (G, H) and blotted (IB) with antiphosphotyrosine. For direct immunoblot the samples were blotted with antibodies anti-phospho-Akt (I, J), antiphospho-JNK (K, L) and anti-phospho-IKKβ (M, N) and anti-phospho-IRS-1 (Ser307) (O, P); $n = 6$. Specific bands were quantified by densitometric analysis. In all experiments, $P < 0.05$; *, vs. saline; &, vs. insulin; $, vs. PTP1B-ASO; Š, vs. PTP1B-ASO + insulin; €, vs. TNF-α; *f*, vs. TNF-α + PTP1B-ASO. Results are presented as means ± S.E.M.

duced IR, IRS-1 tyrosine phosphorylation, and Akt serine phosphorylation. The reduced hypothalamic PTP1B only partially reversed the effect of TNF-α on insulin signaling, at the level of insulin-induced phosphorylation/activation of IR, IRS-1, and Akt (Fig. 2E–J).

Activation of the c-Jun N-terminal kinase (JNK) and IKKβ by TNF-α inhibits insulin signaling, at least in part, by stimulating phosphorylation of insulin receptor substrate 1 (IRS-1) at Ser^{307}. We then investigated whether these mechanisms were also involved in the effect of TNF-α on the insulin signaling pathway.

Our results showed that TNF-α activates JNK and IKKβ. There were no differences in the insulin-stimulated phosphorylation of JNK, IKKβ and IRS-1-Ser307 of rats treated with PTP1B-ASO as compared with rats that were not treated (Fig. 2K–P).

4.3. Activation of leptin action by hypothalamic reduction of PTP1B in the presence of TNF-α

We evaluated the effects of the reduction of the hypothalamic PTP1B, in the presence of TNF-α, on physiological and molecular events controlled by leptin. For this purpose, rats were icv cannulated and, after the recovery period, were treated for 4 days with PTP1B-ASO or saline. On the 4th day both groups were treated with 10^{-6} M leptin alone or TNF-α, followed by leptin. Control rats consumed 21.1 ± 0.5 g of chow. As shown in Fig. 3A, leptin promoted significant reductions in spontaneous food intake to 3.5 ± 1.0 g ($P < 0.05$ compared with saline). The administration of TNF-α, followed by leptin, attenuated leptin-inhibition of food intake to 13.3 ± 0.6 g ($P < 0.05$ compared with leptin – Fig. 3A). Fig. 3B shows that the treatment with PTP1B-ASO decreased food intake (10 ± 0.8 g) and potentiated the leptin-induced suppression of food ingestion to 3.0 ± 0.7 g ($P < 0.05$ compared with leptin). In rats treated with PTP1B-ASO, TNF-α infusion completely blunt at the leptin effect, suggesting that TNF-α does not require PTP1B to fully attenuate leptin's effect (Fig. 3B).

The infusion of leptin increased Tyr-phosphorylation of Jak-2 and Stat3. The co-injection of TNF-α with leptin attenuated leptin-stimulated Jak-2 and Stat3 tyrosine phosphorylation (Fig. 3C and D). We next explored whether the deleterious effect of TNF-α may be reversed through the reduction of hypothalamic PTP1B. Rats treated with PTP1B-ASO showed a significant increase in basal and leptin-induced Jak-2 and Stat3 phosphorylation (Fig. 3E and F). The reduction of hypothalamic PTP1B only partially reversed the effect of TNF-α on leptin signaling at the level of Jak-2 and Stat3 tyrosine phosphorylation (Fig. 3E and F).

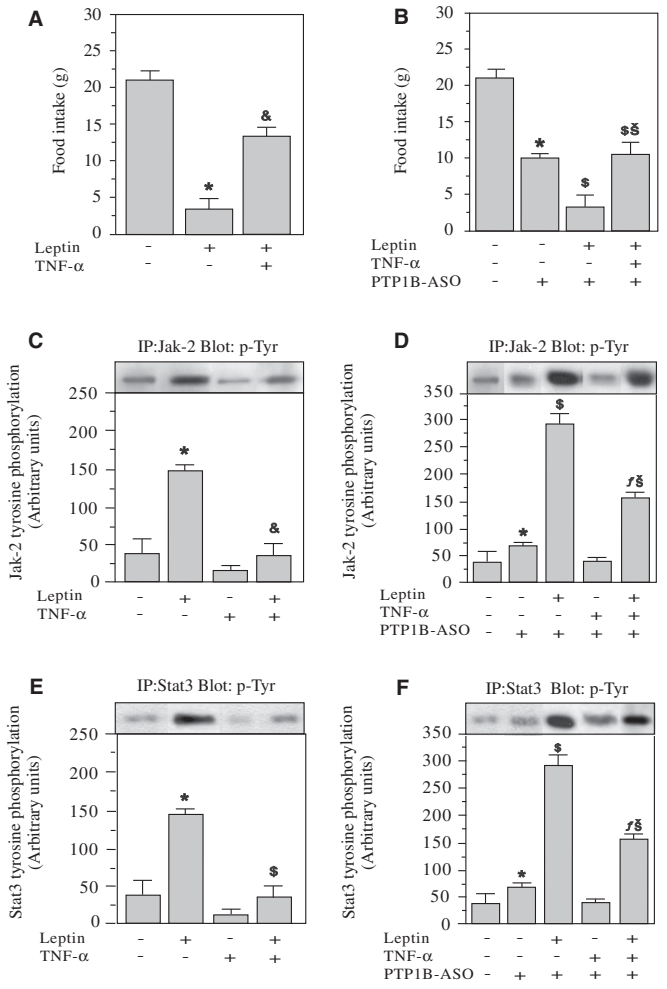

Fig. 3. Activation of leptin action by hypothalamic reduction of PTP1B in the presence of TNF-α. Food ingestion was determined after icv infusion with TNF-α (2.0 μl, 10^{-12} M), leptin (2.0 μl, 10^{-6} M) or leptin plus TNF-α in rats pre-treated with saline (A) or PTP1B-ASO (B); $n = 15$. To evaluate the leptin signaling, rats pre-treated with PTP1B-ASO or saline for 4 days were anesthetized and subjected to craniotomy 15 min after the initial leptin (10^{-6} M) or saline icv infusion and 4 h after the initial TNF-α icv infusion; $n = 6$. The hypothalami were obtained for protein extract preparation. The samples were immunoprecipitated (IP) with antibodies anti Jak-2 (C, E) and Stat3 (D, F) and blotted (IB) with antiphosphotyrosine. Specific bands were quantified by densitometric analysis. In all experiments, $P < 0.05$; *, vs. saline; &, vs. leptin; $, vs. PTP1B-ASO; Š, vs. PTP1B-ASO + leptin; *f*, vs. TNF-α + PTP1B-ASO. Results are presented as means ± S.E.M.

5. Discussion

In the present study, we show that icv administration of TNF-α increases PTP1B protein and activity in the hypothalamus, together with reductions in insulin and leptin sensitivity and signaling. We also show that TNF-α infusion increases phosphorylation of serine residues and decreases tyrosine phosphorylation of PTP1B, which may have a role in the control of PTP1B activity [29]. Our data also reinforce the idea that TNF-α-induced insulin and leptin resistance in hypothalamus, and that the inhibition of hypothalamic PTP1B increases leptin/insulin signaling.

Leptin promotes inhibition of food intake, and PTP1B reduction significantly increases leptin-induced suppression of food ingestion. The administration of TNF-α alone promoted no change in food intake, however, TNF-α followed by leptin significantly reduced the anorexigenic effects of leptin. PTP1B controls leptin signaling through dephosphorylation and inactivation of Jak-2 [8]. Our findings demonstrate that the increases in PTP1B expression and activity in the hypothalamus of rats icv-treated with TNF-α were associated with a decrease in Jak-2 tyrosine phosphorylation levels. Since Jak-2 is a kinase that can mediate many steps downstream from leptin signaling, it is expected that a reduction in Jak-2 phosphorylation will blunt leptin-induced Stat3 phosphorylation. In this regard, our data showed that the leptin resistance, induced by TNF-α, in the rat hypothalamus was also characterized by a less evident increase in Stat3 tyrosine phosphorylation induced by leptin. These alterations were only partially reversed in TNF-α-infused rats that were previously treated with PTP1B-ASO.

Similarly to leptin, insulin also inhibits food intake, and the reduction in PTP1B significantly increased this insulin effect. Although the administration of TNF-α alone did not change food intake, TNF-α followed by insulin significantly reduced the insulin anorexigenic effect. At the molecular level, there was a decrease in insulin-induced IR tyrosine phosphorylation in the hypothalami of TNF-α treated rats. Downstream, there was also a reduction in insulin-induced IRS-1 tyrosine phosphorylation. These alterations may have an important role in insulin resistance in hypothalami, induced by TNF-α. Although, in obese animals, there is an increase in IRS-1 serine phosphorylation in the hypothalamus [30,31], which can contribute to insulin resistance, our data show that the treatment of DIO rats with PTP1B-ASO reverses insulin resistance in the hypothalamus of obese rats and also improves insulin signaling in control rats [4]. Since IRS-1 is the substrate of the insulin receptor, it is possible that the down regulation of PTP1B improved insulin receptor tyrosine kinase activity, culminating with an increase in insulin-induced IRS-1 tyrosine phosphorylation levels and in Akt serine phosphorylation. Several studies have demonstrated that insulin resistance of obesity is mediated by multiple

mechanisms [30,31] and also insulin resistance induced by TNF-α seems to be mediated by more than one mechanism [28]. Previous data show that the lack of PTP1B confers protection against TNF-α-induced insulin resistance [32]. Nieto-Vazquez et al., 2007, demonstrated that, in PTP1B-deficient myocytes, chronic exposure to TNF-α does not impair insulin-induced glucose uptake or insulin signaling. In addition, in PTP1B-/-mice, TNF-α is not able to induce insulin resistance. It is important to mention that this protection was observed in tissues other than the hypothalamus and with higher doses of TNF-α. In the present study we investigated the effect of a very low dose of TNF-α in the hypothalamus of animals previously treated with PTP1B-ASO. We may speculate that we did not observe a clear effect of TNF-α through PTP1B, mainly in consequence of the dose of TNF-α that we used. It is possible that the ability of low doses of TNF-α to induce insulin resistance is independent of PTP1B.

An important mechanism of insulin resistance in different tissues is an increase in IRS-1 serine phosphorylation, specifically at Ser^{307} [31]. This serine phosphorylation can be induced by many serine kinases, but JNK and IKKβ are certainly central in this process and can be activated by TNF-α [17]. Our data showed that, after icv infusion of a low dose of TNF-α there was a clear increase in JNK and, IKKβ phosphorylation and in the parallel in IRS-$1Ser^{307}$ in the hypothalamus. These alterations may have an important role in TNF-α-induced insulin resistance in the hypothalamus, and also the insulin leptin resistance induced by this cytokine.

In conclusion, the results of the present study demonstrated that the inhibition of hypothalamic PTP1B, induced by icv-treatment with PTP1B-ASO, increased leptin/insulin signaling. The icv infusion of a low dose of TNF-α-induced an increase in PTP1B protein expression/activity and attenuated insulin/leptin sensitivity/signaling in the hypothalamus. However, TNF-α was able to completely blunt the leptin and insulin effect in rats treated with PTP1B-ASO. These data suggest that, at least at this low dose, TNF-α does not require PTP1B to fully attenuate the effects of leptin and insulin. In addition, the data of the present study also show that other mechanisms of insulin and leptin resistance such as an increase in IRS-$1Ser^{307}$, are also activated in the hypothalamus by TNF-α.

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