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ARTICLE

New emerging role of protein-tyrosine phosphatase 1B in the regulation of glycogen metabolism in basal and TNF-α-induced insulin-resistant conditions in an immortalised muscle cell line isolated from mice

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Abstract
Aims/hypothesis Protein-tyrosine phosphatase 1B (PTP1B) negatively regulates insulin action, promoting attenuation of the insulin signalling pathway. The production of this phosphatase is enhanced in insulin-resistant states, such as obesity and type 2 diabetes, where high levels of proinflammatory cytokines (TNF-α, IL-6) are found. In these metabolic conditions, insulin action on glycogen metabolism in skeletal muscle is greatly impaired. We addressed the role of PTP1B on glycogen metabolism in basal and insulin-resistant conditions promoted by TNF-α.
Methods We studied the effect of TNF-α in the presence and absence of insulin on glycogen content and synthesis, glycogen synthase (GS) and glycogen phosphorylase (GP) activities and on glycogen synthesis and degradation signalling pathways. For this purpose we used immortalised cell lines isolated from skeletal muscle from mice lacking PTP1B.
Results Absence of PTP1B caused activation of GS and GP with a net glycogenolytic effect, reflected in lower amounts of glycogen and activation of the glycogenolytic signalling pathway, with higher rates of phosphorylation of cyclic adenosine monophosphate-dependent kinase (PKA), phosphorylase kinase (PhK) and GP phosphorylation. Nevertheless, insulin action was strongly enhanced in *Ptp1b* (also known as *Ptpn1*)$^{-/-}$ cells in terms of glycogen content, synthesis, GS activation rates and GS Ser641 dephosphorylation. Treatment with TNF-α augmented the activity ratios of both GS and GP, and impaired insulin stimulation of glycogen synthesis in wild-type myocytes, whereas *Ptp1b*$^{-/-}$ myocytes restored this inhibitory effect. We report a glycogenolytic effect of TNF-α, as demonstrated by greater activation of the degradation signalling cascade PKA/PhK/GP. In our model, this effect is mediated by the activation of PKA.
Conclusions/interpretation We provide new data about the role of PTP1B in glycogen metabolism and confirm the beneficial effect that absence of the phosphatase confers against an insulin-resistant condition.

Keywords Glycogen metabolism · Insulin resistance · Muscle cell · Proinflammatory cytokines · PTP1B · TNF-α

Abbreviations

GP	Glycogen phosphorylase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3

This study is dedicated to the memory of M. Lorenzo, who passed away on 7 April 2010 aged 51 years.

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IR	Insulin receptor
p70s6K	Ribosomal protein S6 kinase, polypeptide 1
PhK	Phosphorylase kinase
PKA	Cyclic adenosine monophosphate-dependent kinase
PKB	Protein kinase B
PKA C	PKA-catalytic subunit C
PTP1B	Protein-tyrosine phosphatase 1B

Introduction

In recent years, many efforts have been made to understand the intricate ways in which glucose metabolism is controlled. Insulin plays a central role in the maintenance of glucose homeostasis, and its function in stimulating glycogen deposition is essential for this purpose [1]. Glycogen is the primary reservoir of glucose in mammalian cells, and its accumulation and degradation is tightly regulated by different factors that mainly modulate rate-limiting enzymes in glycogen metabolism: glycogen synthase (GS) and glycogen phosphorylase (GP) [2–4]. Under anabolic conditions, insulin triggers signalling mechanisms, such as glycogen synthase kinase 3 (GSK3) inactivation and protein phosphatase 1 activation, that lead to dephosphorylation and stimulation of GS [5–9]. As a result, glycogen synthesis is stimulated. Insulin resistance, or the diminished response of cells to insulin action, is the main physiological feature in the onset of type 2 diabetes. Its development is greatly enhanced by the chronic presence of several risk factors (for example, obesity and ageing). One remarkable fact observed in these diseases is the decrease of glycogen synthesis and content [10–12] and the reduced ability of insulin to activate GS [13, 14]. Obesity is a major risk factor for the development of type 2 diabetes, basically because of the altered secretion pattern of adipokines from adipose tissue in the obese state, which may influence insulin sensitivity [15].

As our group has described, TNF-α has been positioned as a link between obesity and insulin resistance [16–18]. Our studies in primary neonatal myotubes highlighted the effect of TNF-α on promoting IRS-1 phosphorylation at the residue Ser307, which impairs the insulin signalling pathway [19]. Nevertheless, only a few studies mention a role for TNF-α in glycogen metabolism [20, 21]. One essential step in insulin signalling is tyrosine residue phosphorylation of its specific insulin receptor (IR) and their substrates (IRSs). Thus, regulation of tyrosine kinase and phosphatase activities seems to be crucial to insulin action. One of the best-characterised phosphatases, protein-tyrosine phosphatase 1B (PTP1B), dephosphorylates tyrosine residues of IR and IRS-1 with the subsequent attenuation of the insulin signalling cascade [22, 23]. Interestingly, the activity of PTP1B and mRNA level of *PTP1B*/*Ptp1b* (also known as *PTPN1*/*Ptpn1*) are elevated in muscle and adipose tissue of obese and diabetic humans and rodents [24, 25] and, as our group has observed, TNF-α and IL-6 action enhance PTP1B levels [26–28]. Moreover, PTP1B overproduction in L6 muscle cells impairs glucose uptake and insulin-stimulated IRS-1 phosphorylation [29–31]. As the role of PTP1B on glycogen metabolism in insulin resistance has not been established, we decided to analyse its effect on glycogen synthesis and content, enzymatic activities (GS and GP) and the most remarkable steps of the signalling pathway. For this purpose, we used immortalised myocyte cell lines from wild-type and PTP1B-deficient neonatal mice generated in our laboratory [28] and we determined the differences between cell types both in basal and insulin-resistant states induced by treatment with the cytokine TNF-α.

Methods

Cell culture PTP1B-deficient and wild-type myocytes were obtained from muscles dissected from $Ptp1b^{+/+}$ and $Ptp1b^{-/-}$ neonates of 3–5 days old (Abbott Laboratories, Chicago, IL, USA) [31]. All procedures were approved by the local ethics committee and performed in accordance with the Federation of European Laboratory Animal Science. Immortalised cell lines were cultured in DMEM–10% fetal calf serum until reaching 90% confluence, moved to serum-free and low-glucose DMEM (10 nmol/l) supplemented with 0.2% (wt/vol.) BSA in the presence or absence of 2 nmol/l TNF-α (Preprotec, Rocky Hill, NJ, USA) for 6 h and further stimulated or not with insulin (Sigma Chemical, St Louis, MO, USA). Shorter periods of insulin stimulation (30–120 min) were used to analyse the enzymatic activities and phosphorylation (which show relatively rapid changes with insulin exposure), while longer incubation times (6 h) were necessary in order to reach sufficient amounts of glycogen in the myocytes for measurement of glycogen content.

Glycogen content determination Cell monolayers were scraped in 30% (wt/vol.) KOH. Homogenates were spotted onto Whatman 3MM paper, and glycogen was precipitated in ice-cold 66% (vol./vol.) ethanol. The papers were incubated in 0.4 mol/l acetate buffer, pH 4.8, with 0.833 mg/ml α-amyloglucosidase (Sigma) for 120 min at 37°C. Glucose release was measured with a glucose kit from Biosystems (Barcelona, Spain).

Glycogen synthesis analysis Cells were incubated with 10 mmol/l [U-^{14}C]glucose (1.85 kBq/μmol) in the presence

or absence of different doses of insulin (0.1–100 nmol/l). The cell monolayer was scraped in 30% (wt/vol.) KOH. Homogenates were spotted onto Whatman 3MM paper and ice-cold 66% (vol./vol.) ethanol was used to precipitate glycogen. The radioactivity of dried papers was counted in a β-radiation counter.

Glycogen degradation analysis Myocytes were incubated in the presence of 10 mmol/l [U-^{14}C]glucose (1.85 kBq/μmol) and insulin 100 nmol/l for 6 h. Then the PKA inhibitor H-89 (10 μmol/l) was added to the medium for 1 h as required by the experiment protocol. Medium was replaced and myocytes were incubated in presence or absence of TNF-α 2 nmol/l, insulin 100 nmol/l or H-89 10 μmol/l. Culture medium was harvested and cell plates were frozen at different times (1–24 h). The cell monolayer was scraped in 30% (wt/vol.) KOH. Homogenates were spotted onto Whatman 3MM paper, glycogen was precipitated in ice-cold 66% (vol./vol.) ethanol and the radioactivity of dried papers was counted (with a β-radiation counter).

GS and GP enzyme activities Cell monolayers were scraped in homogenisation buffer (10 mmol/l Tris/HCl, pH 7.0, 150 mmol/l KF, 15 mmol/l EDTA, 600 mmol/l sucrose, 15 mmol/l 2-mercaptoethanol, 17 μg/l leupeptin, 1 mmol/l benzamidine and 1 mmol/l PMSF). GP activity was determined by the incorporation of [U-^{14}C]glucose-1-phosphate into glycogen in the presence or absence of the allosteric activator AMP (1 mmol/l). GS activity was measured by the incorporation of UDP-[U-^{14}C]glucose in the presence or absence of 10 mmol/l glucose 6-phosphate.

Immunoprecipitation Protein from cell lysates, 500 μg samples, were immunoprecipitated overnight at 4°C with anti-phosphoserine and anti-phospho-IRS-1-specific antibodies from Millipore (Chicago, IL, USA). The resulting immune complexes were collected on agarose beads and subjected to Western blot analyses.

Western blotting We performed Western blots [21] using antibodies against phosphorylated muscle (Ser641) and total GS, phospho-GSK3α/β (Ser21/9), phospho-protein kinase B (PKB) (Ser473), phospho-ribosomal protein S6 kinase, polypeptide 1 (p70s6K; Thr389), phospho-PKA-catalytic subunit C (PKA-C) (Thr197), with all proteins supplied by Cell Signalling (Danvers, MA, USA), anti-phosphoserine (AB1603) from Millipore (Chicago, IL, USA), muscle/brain GP proteins from Santa Cruz Biotechnology (Palo Alto, CA, USA) and the α-subunit of muscle phosphorylase kinase protein from Abcam (Cambridge, UK). Visualisation of immunoreactive bands was carried out using the enhanced chemiluminescence (ECL-Plus) Western blotting protocol (GE Healthcare, Chalfont St Giles, UK).

Data analysis Results are means ± SE from four to ten independent experiments. Statistical significance was tested with a one-way ANOVA followed by the protected least-significant difference test; $p<0.01$were considered significant. In experiments using X-ray films (Hyperfilm; GE Healthcare), different exposure times were used to ensure that bands were not saturated.

Results

Ptp1b$^{-/-}$ myocytes showed higher metabolic rates, glycogen cycling processes and lower glycogen content, and PTP1B absence enhanced insulin sensitivity First, we analysed whether the absence of PTP1B had any effect on glycogen stores in the presence and absence of insulin. As shown in Fig. 1a, *Ptp1b$^{-/-}$* myocytes had lower glycogen content in the basal state (20.96% lower than wild-type myocytes, $p<0.01$). However, the total glycogen reached after insulin stimulation was higher in *Ptp1b$^{-/-}$* myocytes, (10% vs wild type) for the range of doses from 1 to 100 nmol/l. Glycogen stores are strongly correlated with the synthesis/degradation ratio. For this reason, we measured the rate of glucose incorporation into glycogen to analyse glycogen synthesis. Unexpectedly, the rate was already significantly higher in *Ptp1b$^{-/-}$* myocytes in the basal state ($p<0.01$; Fig. 1b). Both *Ptp1b$^{+/+}$* and *Ptp1b$^{-/-}$* cell types strongly responded to insulin action at doses of 10 to 100 nmol/l, but *Ptp1b$^{-/-}$* myocytes reached higher levels of glycogen synthesis (12.88 vs 17.91 nmol incorporated glucose/mg protein).

Next, we investigated GS activity: insulin (100 nmol/l) was added to the cell culture medium and activity measurements were carried out. Both cell types showed a marked increase of GS activation (about 50%), although PTP1B-deficient myocytes showed higher levels of GS activity ratio in basal as well as in insulin-stimulated conditions in contrast to the wild-type myocytes (42% and 26% in each case; Fig. 1c).

Covalent modification of different serine residues of GS strongly modulates enzyme activity. In this regard, insulin enhances GS activation by promoting a decrease in phosphorylation of the enzyme. Thus, we analysed the phosphorylation levels of several enzymes implicated in the insulin signalling cascade and phosphorylation of GS Ser641 (Fig. 1d). After insulin stimulation for 30 min, *Ptp1b$^{-/-}$* myocytes exhibited higher phosphorylation levels of PKB, GSK3α/β and p70s6K and a stronger dephosphorylation of GS Ser641 at low doses of insulin (0.1 nmol/l). Furthermore, *Ptp1b$^{-/-}$* myocytes showed a more sustained action of insulin after 2 h of stimulation, even at low concentrations of 1 nmol/l, whereas wild-type myocytes only responded to the highest concentration of insulin (100 nmol/l; Electronic supplementary material [ESM] Fig. 1).

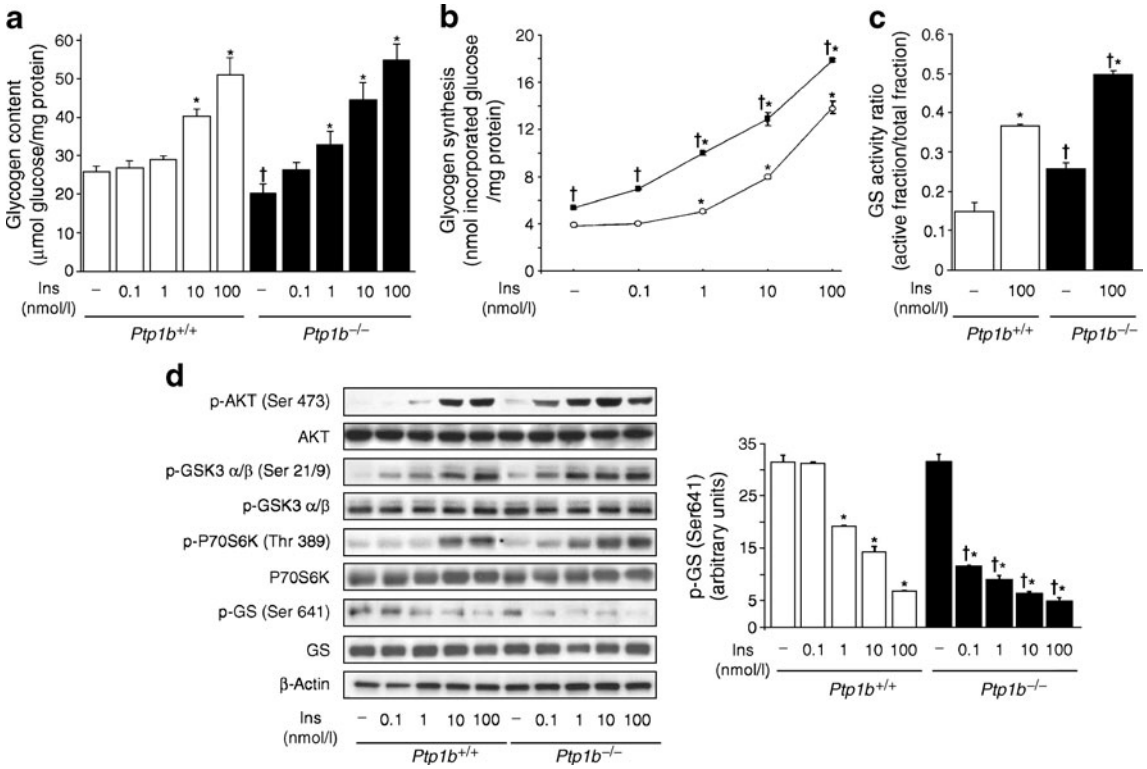

Fig. 1 Dose–response effect on insulin-induced glycogen content, synthesis, GS activity and GS phosphorylation pattern in wild-type and PTP1B-deficient myocytes. **a** *Ptp1b*$^{+/+}$ myocytes (white bars) and *Ptp1b*$^{-/-}$ myocytes (black bars) were stimulated for 6 h with different doses of insulin (0.1–100 nmol/l) and glycogen content was measured. **b** To determine glycogen synthesis, cells were incubated in the presence of 10 mmol/l [U-^{14}C]glucose with different doses of insulin (0.1–100 nmol/l) for 6 h, and the quantity of radioactive glucose incorporated into glycogen in the homogenates was measured; *Ptp1b*$^{+/+}$ myocytes, white circles; *Ptp1b*$^{-/-}$ myocytes, black squares. **c** For the assay for GS activity, *Ptp1b*$^{+/+}$ myocytes (white bars) and *Ptp1b*$^{-/-}$ myocytes (black bars) were stimulated with 100 nmol/l insulin for 30 min. GS activity was determined in cell homogenates as incorporation of radioactivity from UDP-[U-^{14}C]glucose into glycogen in the presence or absence of the allosteric effector glucose 6-phosphate. **d** The insulin signalling pathway was assayed in *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ myocytes after stimulation with different doses of insulin (0.1–100 nmol/l) for 30 min. Total cell extracts were subjected to western blot and immunodetected with antibodies against phospho-PKB (Ser473), phospho-GSK3α/β (Ser21/9) and phospho-p70s6K (Thr389) and total and phosphorylated GS (Ser641) proteins. The autoradiograms were quantified by scanning densitometry. The results are mean ± SE, with n=4 as a minimum. *p<0.01 for test vs basal; †p<0.01 for *Ptp1b*$^{-/-}$ cells vs wild-type cells under the same experimental conditions. Ins, insulin

The glycogen degradation process, along with glycogen synthesis, is relevant in the regulation of the glycogen stores. Therefore, we explored GP activity after treatment with different insulin doses (Fig. 2). Interestingly, the basal GP activity ratio was higher in *Ptp1b*$^{-/-}$ myocytes (Fig. 2a) and insulin treatment decreased the GP activity ratio in both cell types. Next, we studied the pattern of phosphorylation of GP and of its specific kinase, phosphorylase kinase (PhK). Higher rates of phosphorylation of these enzymes turn them to an active state, and insulin promotes a decrease in the phosphorylation rate of these proteins. Immunoblot analyses of GP and PhK phosphoserine immunoprecipitates revealed that increasing doses of insulin promoted similar decreases in GP and PhK phosphorylation in both cell types (Fig. 2b). Surprisingly, we achieved greater GP and PhK phosphorylation levels at the basal states in *Ptp1b*$^{-/-}$ myocytes.

In summary, the data above show that lack of PTP1B establishes a glycogen cycling process with a dual activation of both GS and GP and with a net glycogenolytic effect, reflected by lower glycogen content. In the absence of PTP1B, insulin action was enhanced, mainly in the steps involved in glycogen synthesis.

Absence of PTP1B protects against TNF-α effects on glycogen metabolism Previous studies have described the ability of TNF-α to block insulin action on glucose uptake in skeletal muscle. Contributions made by our group also revealed that TNF-α enhances PTP1B content and activity, and that the absence of the phosphatase protects against the effects of TNF-α on insulin-induced glucose uptake. However, given that just a few studies mention a potential role of this cytokine on glycogen metabolism, we decided to explore its effect at different levels. For this purpose,

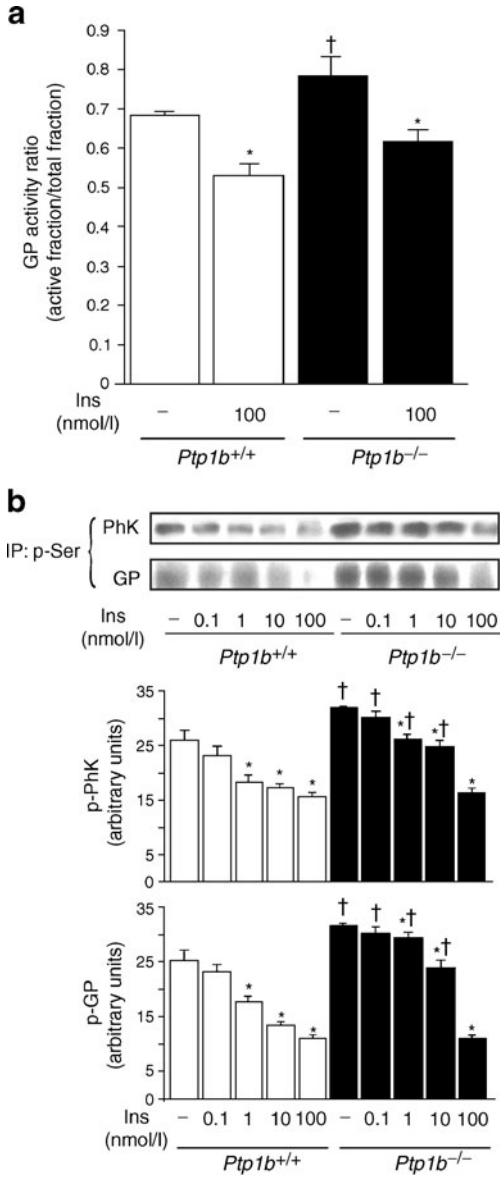

Fig. 2 Insulin action on the glycogen degradation process. **a** GP activity ratio was determined following the protocol described for Fig. 1c, using AMP as the allosteric effector in this case. Myocytes were stimulated with different doses of insulin (0.1–100 nmol/l) for 30 min to assay the rate of serine phosphorylation in PhK. **b** Cell lysates were obtained, and 500 μg samples of protein were immunoprecipitated with a specific antibody against phosphoserine residues. The collected immune complexes were subjected to Western blot and immunodetected with antibodies against GP and PhK. The autoradiograms were quantified by scanning densitometry. Similar results were obtained in four independent experiments. *$p<0.01$ for test vs basal; †$p<0.01$ for *Ptp1b*$^{-/-}$ cells vs wild-type cells under the same experimental conditions. Ins, insulin

myocytes were pretreated with TNF-α (2 nmol/l) for 24 h, and then stimulated with insulin 100 nmol/l for 6 h.

First, we detected a decrease in glycogen content in cells pretreated with TNF-α (18.22 and 12.22 μmol glucose/mg protein in *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ cells, respectively) vs control (25.81 and 20.39 μmol glucose/mg protein in *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ cells, respectively) and a blocking effect on insulin-induced glycogen content in wild-type cells pretreated with TNF-α (Fig. 3a). Interestingly, the TNF-α blocking effect was almost completely abolished in myocytes lacking PTP1B, which reached similar levels of insulin-induced glycogen content as control cells. Similarly, a marked decrease in insulin-induced glycogen synthesis appeared in wild-type myocytes pretreated with TNF-α (Fig. 3b). Once again, this blocking effect was abolished in *Ptp1b*$^{-/-}$ myocytes, and significantly higher levels of insulin stimulation were reached in the presence of TNF-α vs *Ptp1b*$^{+/+}$ myocytes.

Next, we tested the impact of TNF-α on GS activity under insulin stimulation. Our data pointed to an inhibitory role of TNF-α, impairing insulin action on GS activity (Fig. 3c) in wild-type myocytes; meanwhile, no significant effect was detected in *Ptp1b*$^{-/-}$ cells. Both wild-type and PTP1B-deficient myocytes showed a statistically significant increase in GS activation after 24 h of TNF-α treatment, which was more pronounced in *Ptp1b*$^{-/-}$ myocytes ($p<0.01$). Next, we studied insulin action on GS and GSK3α/β phosphorylation and PTP1B total protein content in TNF-α pretreated myocytes. For this purpose myocytes were cultured in presence of TNF-α (2 nmol/l) for 24 h, and then stimulated for 30 min with insulin 100 nmol/l. Insulin promoted significant decreases in GS Ser641 phosphorylation in both cell types, whereas insulin action on GSK3α/β phosphorylation was more evident in *Ptp1b*$^{-/-}$ cells (Fig. 3d).

TNF-α pretreatment considerably impaired insulin action in *Ptp1b*$^{+/+}$ myocytes but not in *Ptp1b*$^{-/-}$ myocytes, which responded strongly to insulin. Moreover, PTP1B protein content was greatly enhanced by TNF-α in wild-type myocytes. Despite the effect of the cytokine in enhancing GS activity ratio (Fig. 3c), TNF-α induced increases in GS Ser641 phosphorylation in both cell types. No changes were observed in total GS protein content in any of the conditions tested.

We also studied the effect of PTP1B absence on IRS-1 phosphorylation rates in serine residues, an effect that is known to be strongly enhanced by TNF-α treatment. TNF-α promoted increases in IRS-1 serine phosphorylation both in wild-type and *Ptp1b*$^{-/-}$ myocytes (Fig. 4), although the basal phosphorylation rate was significantly lower in *Ptp1b*$^{-/-}$ cells. Insulin was able to abolish this TNF-α effect in cells lacking PTP1B but not in wild-type cells. Thus, our data revealed a dual role for TNF-α, enhancing the basal GS activity ratio and blocking insulin action, not only in terms of glucose uptake [27, 28] but also in several steps within the insulin signalling cascade and glycogen synthesis.

Implication of TNF-α in the glycogen degradation process TNF-α strongly increased the GP activity ratio, especially in *Ptp1b*$^{-/-}$ myocytes, in which increases of 44% over basal were achieved (Fig. 5a). In addition, insulin action attenuated the GP activity ratio in both cell

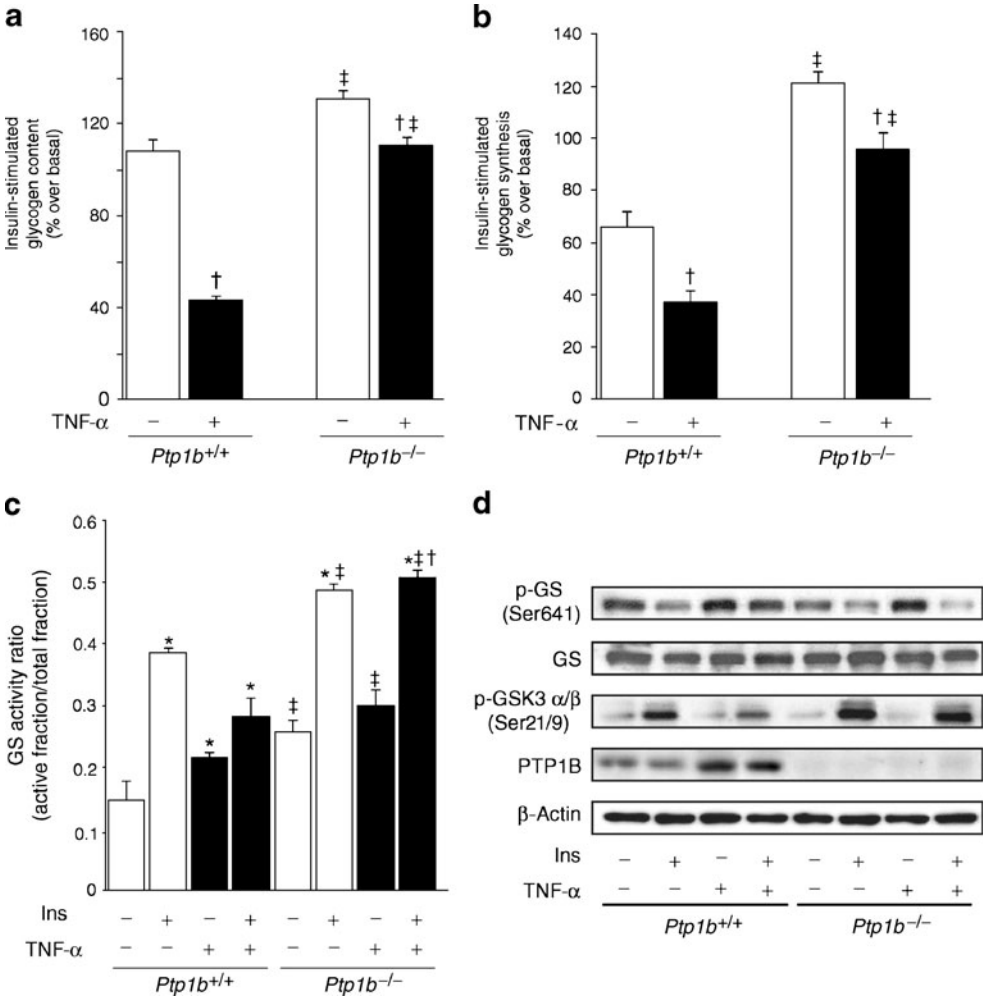

Fig. 3 The effects of TNF-α-induced insulin resistance on glycogen synthesis and content and GS activation are reverted in PTP1B-deficient myocytes. PTP1B-deficient and wild-type myocytes were cultured in serum-free and low-glucose (10 mmol/l) medium for 24 h in the presence or absence of 2 nmol/l TNF-α, stimulated with insulin (100 nmol/l) for 6 h and (**a**) glycogen content and (**b**) glycogen synthesis were determined. Results are the means ± SE of nine independent experiments and are expressed as the percentage of insulin stimulation over the basal level in the presence or absence of TNF-α. Finally, we determined (**c**) the GS ratio activity and (**d**) the GS phosphorylation pattern in the presence or absence of TNF-α 2 nmol/l for 24 h with and without insulin stimulation (100 nmol/l) for 30 min. Control cells were not exposed to the hormone. Total cell extracts were standardised for protein concentration, subjected to Western blot and immunodetected with antibodies against total and phosphorylated GS (Ser641). The autoradiograms were quantified by scanning densitometry. Similar results were obtained in four independent experiments. *$p<0.01$ for test vs basal; †$p<0.01$ for TNF-α plus insulin vs insulin alone; ‡$p<0.01$ for $Ptp1b^{-/-}$ vs $Ptp1b^{+/+}$ myocytes. Ins, insulin

types, in the presence and absence of TNF-α. GP protein content (Fig. 5b) decreased significantly under insulin-stimulated conditions ($p<0.01$). Moreover, TNF-α treatment counteracted this insulin effect only in $Ptp1b^{+/+}$ myocytes.

To further explore the specific mechanism of TNF-α action on glycogen degradation, we examined the regulation of PKA, which mediates glycogenolysis and manifests elevations in threonine phosphorylation under activating stimuli (Fig. 5c). We analysed levels of phosphorylation of PKA-C in Thr197, which reflects the activation rate of the enzyme. We confirm a strong activating effect of TNF-α on PKA-C phosphorylation in which is more pronounced in $Ptp1b^{-/-}$ myocytes. Moreover, the basal PKA-C activation is significantly higher in $Ptp1b^{-/-}$ myocytes. Interestingly, insulin did not counteract the effects of TNF-α on PKA-C in either of the two cell types. The specific role of PKA in TNF-α-induced glycogenolysis was investigated (Fig. 6), using an inhibitor of PKA (H-89) specific to the purpose. Pretreatment with H-89 (10 μmol/l) restored TNF-α-induced glycogen degradation in both cell types (Fig. 6a), and raised glycogen basal levels in $Ptp1b^{-/-}$ myocytes. In addition, insulin abolished TNF-α glycogenolytic effects in the presence or absence of H-89 only in $Ptp1b^{-/-}$ myocytes. Analyses of phosphoserine immunoprecipitates against GP and PhK (Fig. 6b) revealed greater phosphorylation of both

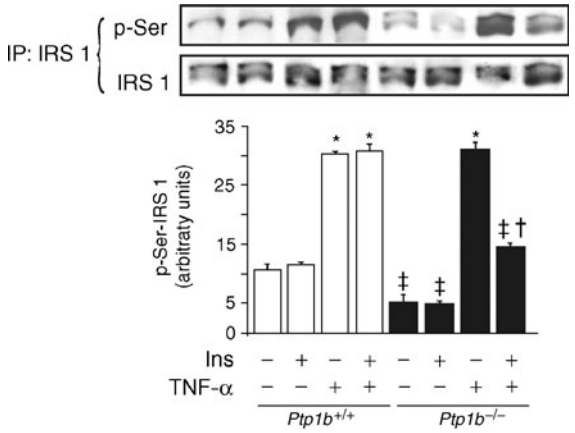

Fig. 4 The absence of PTP1B diminishes TNF-α-induced IRS-1 serine phosphorylation with insulin stimulation. Myocytes were incubated in serum-free and low-glucose (10 mmol/l) medium in the absence or presence of TNF-α 2 nmol/l for 24 h and then stimulated with 100 nmol/l insulin for 30 min. IRS-1 phosphorylation of serine residues was tested. Total cell extracts were standardised for protein concentration, subjected to Western blot and immunodetected with specific antibodies against IRS-1. Similar results were found for four independent experiments. *$p<0.01$ for test vs basal; †$p<0.01$ for TNF-α plus insulin vs insulin alone; ‡$p<0.01$ for PTP1B-deficient cells vs wild-type cells. Ins, insulin

enzymes with TNF-α treatment that was blocked when H-89 was added to the culture medium. Insulin treatment only decreased phosphorylation levels in PTP1B-deficient myocytes pretreated with TNF-α.

Taken together, we reveal a role for TNF-α in glycogen metabolism, affecting both the GS and GP activity ratios and activating the main enzymes implicated in glycogenolysis. This glycogenolytic effect is, in part, mediated by PKA, which seems to be implicated as a cause of the low glycogen content found in *Ptp1b*$^{-/-}$ myocytes in the basal state. We observed a blocking effect of TNF-α on insulin action at various levels in glycogen metabolism that did not occur in myocytes deficient in PTP1B.

Discussion

Insulin is essential in glycogen synthesis regulation and its action is tightly controlled by several mechanisms. One of these mechanisms is carried out by PTP1B, which attenuates the insulin signalling pathway. Our previously published data showed that PTP1B deficiency does not modify protein levels of skeletal muscle markers or proteins involved in the insulin signalling pathway and strong responses to insulin

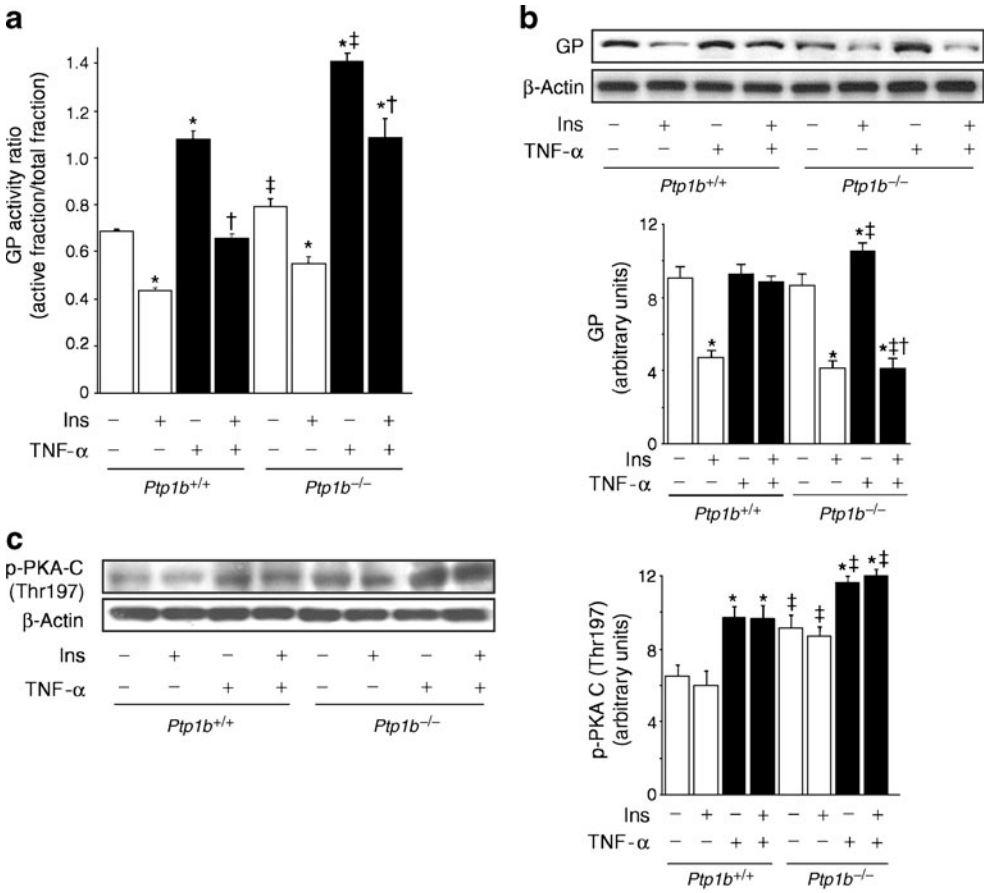

Fig. 5 Glycogenolytic role of TNF-α in *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ myocytes. Myocytes were incubated in serum-free and low-glucose (10 mmol/l) medium in the presence or absence of TNF-α 2 nmol/l for 24 h and were then stimulated with 100 nmol/l insulin for 30 min. The GP activity ratio was assayed (**a**) and GP total content (**b**) and phosphorylated PKA-C (p-PKA-C) (Thr197) (**c**) were analysed. Total cell extracts were subjected to Western blot and were immunodetected with specific antibodies against PhK, GP and p-PKA C (Thr197). Similar results were found for four independent experiments. *$p<0.01$ for test vs basal; †$p<0.01$ for TNF-α plus insulin vs insulin alone; ‡$p<0.01$ for PTP1B-deficient cells vs wild-type cells. Ins, insulin

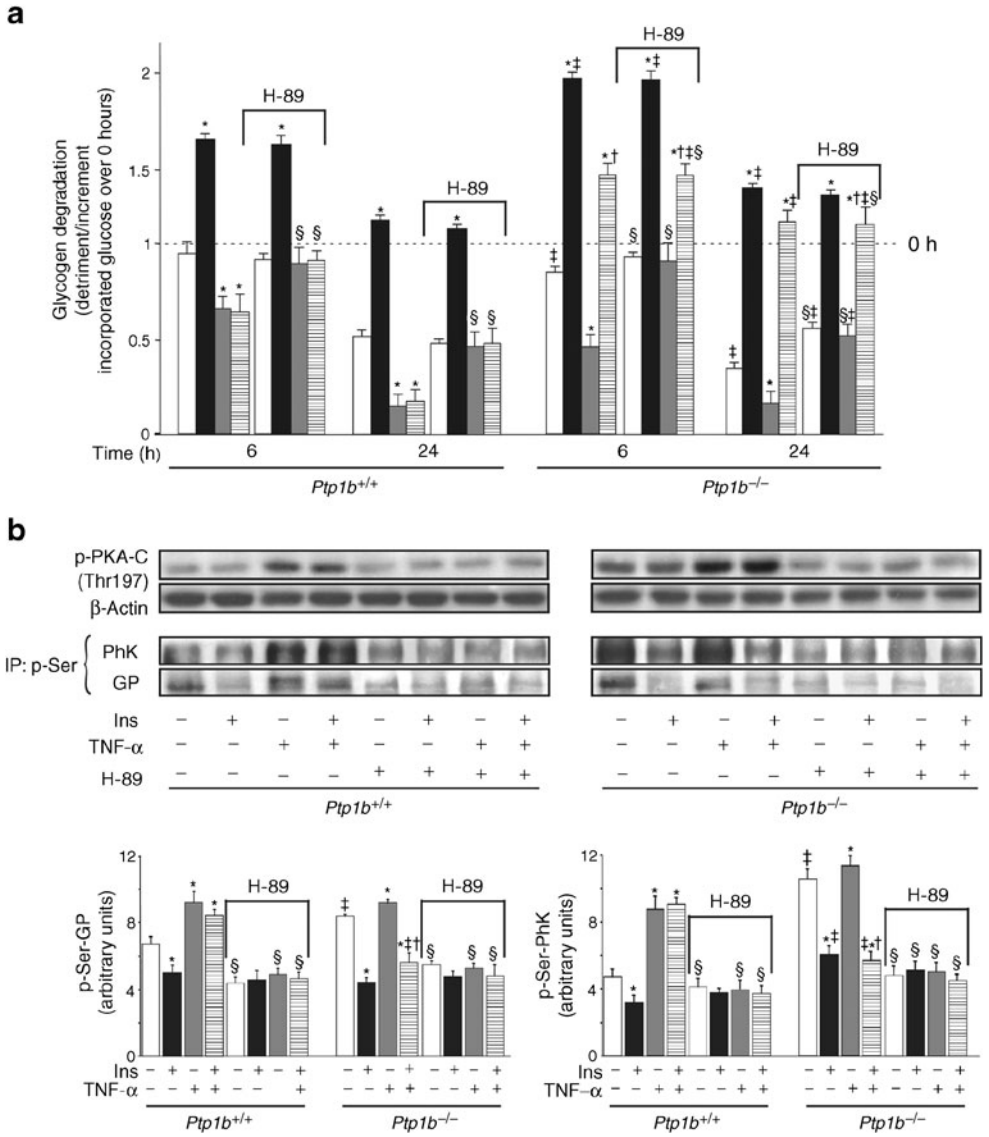

Fig. 6 The specific role of PKA in TNF-α-induced glycogenolysis in *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ myocytes. **a** To determine glycogen degradation, myocytes were incubated in the presence of 10 mmol/l [U-^{14}C] glucose and insulin 100 nmol/l for 6 h, and then H-89 (10 μmol/l) was added to the medium for 1 h as required. Medium was replaced (we consider this point 0 h) and myocytes were incubated in presence or absence of TNF-α 2 nmol/l, insulin 100 nmol/l or H-89 10 μmol/l. Culture medium was harvested and cell plates frozen at different times (1–24 h). The quantity of radioactive glucose incorporated into glycogen in the homogenates was determined and is expressed vs the values of control cells at 0 h. **b** For analysing PKA involvement in TNF-α-induced GP and PhK serine phosphorylation, myocytes were incubated in the presence or absence of H-89 (10 μmol/l) for 1 h and then cultured in serum-free and low-glucose (10 mmol/l) medium for 24 h in the presence or absence of 2 nmol/l TNF-α and were finally stimulated with insulin 100 nmol/l for 30 min. Cell lysates were obtained, and 500 μg samples of protein were immunoprecipitated with a specific antibody against phosphoserine residues. The collected immune complexes and cell lysates were subjected to Western blot and immunodetected with antibodies against phosphorylated PKA-C (p-PKA-C) (Thr197), GP and PhK. The autoradiograms were quantified by scanning densitometry. Similar results were obtained in four independent experiments. White bars, control; black bars, plus insulin; grey bars, plus TNF-α; striped bars, plus insulin and TNF-α. *$p<0.01$ for test vs basal; †$p<0.01$ for TNF-α plus insulin vs insulin alone; ‡$p<0.01$ for PTP1B-deficient cells vs wild-type cells; §$p<0.01$ for the presence of H-89 vs the absence of H-89. Ins, insulin

action are reported in mice lacking this protein [28]. The present study also showed enhanced insulin sensitivity in *Ptp1b*$^{-/-}$ myocytes, with higher rates of insulin-induced glycogen content, glycogen synthesis, activation of GS enzyme and lack of phosphorylation of GS Ser641 observed. Interestingly, we observed lower levels of IRS-1 phosphorylation in serine residues in *Ptp1b*$^{-/-}$ myocytes.

The absence of PTP1B may modulate glycogen synthesis processes, promoting increases in the GS activity ratio, glucose incorporation into glycogen, and also glycogen

degradation mechanisms, raising the GP activity ratio and PKA, PhK and GP phosphorylation in the basal state. The metabolic balance of these events results in a net elevation of glycogenolysis, with lower glycogen stores found in basal conditions. In this regard, Klaman et al. described higher metabolic rates and energy expenditure in *Ptp1b*$^{-/-}$ mice [32]. This fact may be in concordance with data observed in *Ptp1b*$^{-/-}$ myocytes, which suggest higher glycogen degradation rates to satisfy a greater energy demand. The rate of GS activation results from the actions of many factors that may promote covalent modifications or act as allosteric effectors. The GS residue Ser641 is a crucial regulatory site and its phosphorylation (inactivation) or dephosphorylation (activation) pattern depends on external stimuli (insulin, adrenalin) [33]. Also, the amount of glycogen stored within the cell strongly influences GS activity, GS subcellular redistribution and GS phosphorylation patterns, thereby masking the effects promoted by insulin and other factors [34–36] Furthermore, AMP-activated protein kinase (AMPK) acts as a sensor of the glycogen stores within the cell and promotes GS inhibition when glycogen depots increase [37]. Thus, the higher GS activity found in *Ptp1b*$^{-/-}$ myocytes may be a result of the lower glycogen content found in the basal state. Although Ser641 phosphorylation was not affected, it is worth noting that GS is phosphorylated at multiple sites that regulate its activity in addition to Ser641 (Ser645 and Ser7), and it is not completely understood how each site contributes to enzyme activity. In addition, we observed an unexpected increase in the glycogen degradation process and higher activation of the PKA/PhK/GP signalling pathway in *Ptp1b*$^{-/-}$ myocytes that is abolished by pretreatment with a specific PKA inhibitor (H-89). Our results indicate that the absence of PTP1B enhances the entire metabolic process and energy demand, resulting in high glycogen synthesis and degradation rates, even in basal conditions, as well as a strong activation of enzymatic machinery. Furthermore, a glycogen cycle is established with a net glycogenolytic balance, which we observed in previous studies carried out in hepatocytes, where treatment with dexamethasone promoted increases in activation of both GS and GP enzymes [38]. These results provide new information to clarify some features observed in *Ptp1b*$^{-/-}$ mice, such as the high energy expenditure [28, 32].

The molecular mechanisms underlying insulin stimulation of glycogen synthesis are not fully clear and several pathways have been proposed, such as the phosphoinositides 3-kinase/protein kinase B/GSK3 pathway [9] or the mechanistic target of rapamycin (serine/threonine kinase) (mTOR)/p70s6K cascade [39]. Finally, GS Ser641 dephosphorylation reflects insulin-stimulated GSK3 phosphorylation [40]. Our model achieved good responses to insulin action with regard to glycogen synthesis and content, GS activation, GSK3α/β phosphorylation and GS Ser641 dephosphorylation, and PTP1B deficiency enhanced these responses. Insulin also exerted an inhibitory effect on GP activity ratio; however, this was not potentiated in *Ptp1b*$^{-/-}$ myocytes. PTP1B deficiency alone may enhance GP activity thus counteracting insulin effects. Moreover, the higher metabolic rates showed by *Ptp1b*$^{-/-}$ myocytes may increase ATP consumption and AMP production. AMP exerts a strong allosteric effect inducing GP activity independently of covalent modifications [41, 42]. Otherwise, other kinases such as PKA could lead to strong activation of the enzyme. Although previous studies have already reported higher rates of PKA activation in muscle and adipose tissue of *Ptp1b*$^{-/-}$ mice [43], our findings reveal that PKA activation (expressed in terms of PKA-C activation) remains higher even in the presence of insulin in *Ptp1b*$^{-/-}$ myocytes and that this increment is associated with the low glycogen content observed in the basal state. In this regard, H-89 pretreatment raises glycogen levels in basal conditions. We can suggest that the cAMP/PKA signalling pathway is much more active in PTP1B-deficient mice, confirming the relevance of PTP1B in regulating various processes within the cell. Our studies also revealed a novel role for insulin acting at the GP protein stability level, or encouraging the early degradation of GP. In this sense, several mechanisms of protein turnover, such as the ubiquitin–proteasome pathway [44, 45], or calcium-dependent proteases such as calpain [46], are known to regulate insulin action. GP has been identified as a natural calpain target, so this protease may regulate GP activity and degradation [47]. The underlying mechanism remains to be elucidated. Thus, PTP1B emerges as a new player in the regulation of glycogen metabolism, so that its absence triggers higher metabolic rates and lower amounts of glycogen. Moreover, the glycogen enzymatic machinery (GS and GP) is significantly activated in the absence of PTP1B, and the net result of this glycogen cycle leads to a strong glycogenolytic effect. In addition, insulin action is enhanced in PTP1B-deficient myocytes.

Several studies have proposed TNF-α as a link between insulin resistance and obesity [48] and, as our group has previously described, both adipocytes and myocytes showed defects at different levels in insulin action after chronic exposure to the cytokine [16, 27]. Nevertheless, few data on the role of TNF-α on glycogen metabolism have emerged in recent years [49]. Our data show that TNF-α strongly impairs insulin action on glycogen synthesis and glycogen content, GS activity and GS Ser641 phosphorylation, and enhances IRS-1 phosphorylation of serine residues in wild-type myocytes. We also observed enhanced PTP1B protein content in wild-type myocytes pretreated with TNF-α [28]. Moreover, PTP1B seems to play a crucial role in these mechanisms as its absence

restores insulin effects in the presence of TNF-α. In fact, in the presence of insulin, PTP1B deficiency prevented the increase of IRS-1 serine phosphorylation promoted by TNF-α. In previous studies, *Ptp1b*$^{-/-}$ myocytes showed higher tyrosine phosphorylation rates in IR and IRSs [28]. This may block the effect of TNF-α on IRS-1 phosphorylation through conformational constraints within the protein. In addition, triggering of other signalling events could interfere with the kinases required for TNF-α-induced phosphorylation of IRS-1 in serine residues (for example, mitogen-activated protein kinase 8 [JNK], IκB kinase [IKK], suppressors of cytokine signalling [SOCS]). Further studies on this topic are required.

TNF-α also promotes increases in GS activity, which could be due to the higher rates of glucose uptake induced by the cytokine that we have reported in our studies [17, 28]. Chronic exposure to TNF-α increases expression levels of *Glut1* (also known as *Slc2a1*), which enhances glucose uptake and glucose 6-phosphate production within the cell. Glucose 6-phosphate acts as a powerful allosteric effector of GS activity. So far, TNF-α involvement in the glycogenolysis process has not been reported. In our study TNF-α strongly induces glycogenolysis both in wild-type and *Ptp1b*$^{-/-}$ myocytes, with increases in the GP activity ratio occurring even in the presence of insulin. As indicated in this work, TNF-α somehow induces PKA by increasing phosphorylation in Thr197 PKA-C, which results in stimulation of GP activity. In this regard, TNF-α was shown to increase intracellular levels of cAMP and the active form of PKA in human adipocytes [50]. PKA specifically phosphorylates PhK, which activates GP by phosphorylation of serine residues. In our study, TNF-α induced phosphorylation of PKA-C, PhK and GP and enhanced glycogen degradation through PKA activation. In fact, specific PKA inhibition with H-89 blocked the TNF-α glycogenolytic effect on glycogen degradation and PhK/GP phosphorylation patterns. However, in the absence of PTP1B, TNF-α did not further phosphorylate GP and PhK, despite having an activating effect on GP. Moreover, in *Ptp1b*$^{-/-}$ cells, insulin was able to abrogate the TNF-α-promoted activation of GP and PhK, but PKA-C remained activated.

In summary, we describe a novel effect of TNF-α on glycogen synthesis in basal states, where substantial GS activation is promoted by the cytokine. Despite this effect, TNF-α diminishes insulin action on glycogen synthesis, content and GS activation, probably through its interfering effect on the insulin signalling pathway. TNF-α also triggers signalling events that involve PKA activation that ultimately lead to strong GP stimulation, with the subsequent elevation of glycogenolysis. We have described a new role for insulin on GP regulation, thus the hormone may be regulating its stability at protein levels. The absence of PTP1B enhances the metabolic rate, with low glycogen content and the presence of a glycogen cycle in which both GP and GS are activated, with a net glycogenolytic effect. Furthermore, the absence of PTP1B enhanced insulin sensitivity and restored insulin action in myocytes pre-treated with TNF-α, thus confirming the protective role of PTP1B against insulin resistance mediated by this cytokine.

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