Inducible liver-specific knockdown of protein tyrosine phosphatase 1B improves glucose and lipid homeostasis in adult mice

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Abstract

Aims/hypothesis Protein tyrosine phosphatase 1B (PTP1B) is a key negative regulator of insulin signalling. Hepatic PTP1B deficiency, using the *Alb-Cre* promoter to drive *Ptp1b* deletion from birth in mice, improves glucose homeostasis, insulin sensitivity and lipid metabolism. The aim of this study was to investigate the therapeutic potential of decreasing liver PTP1B levels in obese and insulin-resistant adult mice.

Methods Inducible *Ptp1b* liver-specific knockout mice were generated using *SA-Cre-ER^{T2}* mice crossed with *Ptp1b* floxed (*Ptp1b^{fl/fl}*) mice. Mice were fed a high-fat diet (HFD) for 12 weeks to induce obesity and insulin resistance. Tamoxifen was administered in the HFD to induce liver-specific deletion of *Ptp1b* (*SA-Ptp1b^{-/-}* mice). Body weight, glucose homeostasis, lipid homeostasis, serum adipokines, insulin signalling and endoplasmic reticulum (ER) stress were examined.

Results Despite no significant change in body weight relative to HFD-fed *Ptp1b^{fl/fl}* control mice, HFD-fed *SA-Ptp1b^{-/-}* mice exhibited a reversal of glucose intolerance as determined by improved glucose and pyruvate tolerance tests, decreased fed and fasting blood glucose and insulin levels, lower HOMA of insulin resistance, circulating leptin,

serum and liver triacylglycerols, serum NEFA and decreased HFD-induced ER stress. This was associated with decreased glycogen synthase, eukaryotic translation initiation factor-2 α kinase 3, eukaryotic initiation factor 2 α and c-Jun NH₂-terminal kinase 2 phosphorylation, and decreased expression of *Pepck*.

Conclusions/interpretation Inducible liver-specific PTP1B knockdown reverses glucose intolerance and improves lipid homeostasis in HFD-fed obese and insulin-resistant adult mice. This suggests that knockdown of liver PTP1B in individuals who are already obese/insulin resistant may have relatively rapid, beneficial therapeutic effects.

Keywords Disease · ER stress · Glucose · Insulin · Leptin · Lipid · Liver · NAFLD · Phosphatase · PTP1B

Abbreviations

ALT	Alanine aminotransferase
eIF2 α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3
GTT	Glucose tolerance test
HFD	High-fat diet
HOMA-IR	HOMA of insulin resistance
IR	Insulin receptor
JAK2	Janus kinase 2
JNK	c-Jun NH ₂ -terminal kinase
MCP-1	Monocyte chemoattractant protein 1
mTOR	Mammalian target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
PERK	Eukaryotic translation initiation factor-2 α kinase 3

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PKB	Protein kinase B
PP2A	Protein phosphatase 2
PTP1B	Protein tyrosine phosphatase 1B
PTT	Pyruvate tolerance test
SHP2	SH2 domain-containing protein tyrosine phosphatase 2
WAT	White adipose tissue

Introduction

Caloric excess and low physical activity are key drivers of rising obesity levels in Western society. Insulin resistance and obesity are associated with the development of cardiovascular disease, type 2 diabetes and cancer [1]. Insulin resistance leads to hyperglycaemia, caused by a decrease in insulin-dependent glucose uptake into peripheral tissues and diminished ability of insulin to suppress hepatic glucose production [2]. Insulin resistance is also associated with dyslipidaemia [3] and non-alcoholic fatty liver disease (NAFLD), which is the most common liver disease throughout the world (20–35% of the population) [4]. It is distinguished by excess hepatic fat stores, in the absence of alcohol consumption [4]. Lifestyle factors such as nutrition and exercise can influence whether NAFLD is likely to progress to non-alcoholic steatohepatitis [4], which carries an increased risk of mortality [5]. This rising burden of metabolic disease requires the development of new therapeutic strategies [6].

Protein tyrosine phosphatase 1B (PTP1B; also known as PTPN1) is a non-receptor tyrosine phosphatase which is ubiquitously expressed in all insulin-responsive tissues [7]. PTP1B is a negative regulator of both insulin and leptin signalling through its actions on the insulin receptor (IR) and Janus kinase 2 (JAK2) [7]. Whole-body *Ptp1b*^{−/−} mice have enhanced insulin sensitivity, increased phosphorylation of tyrosine residues on the IR and reduced adiposity on a high-fat diet (HFD) [8, 9]. PTP1B antisense oligonucleotides were shown to effectively lower PTP1B levels in liver and fat, enhance insulin signalling and decrease adiposity in *ob/ob* and *db/db* mice [10, 11]. Lipogenic genes were downregulated in fat and liver, including diminished *Pparg* expression in adipose tissue [12]. Whether these positive effects were due to loss of PTP1B in the liver and/or adipose tissue or any other tissue(s) was, however, unclear. Subsequently, tissue-specific *Ptp1b* knockout mice were generated to identify the specific tissues responsible for PTP1B's effects on insulin sensitivity and lipid metabolism [2, 13, 14].

On an HFD, neuronal *Ptp1b*^{−/−} mice display decreased body mass, reduced food intake and enhanced energy expenditure [13]. This is due, at least in part, to leptin hypersensitivity in these mice; PTP1B acts as a negative regulator of leptin signalling via its ability to dephosphorylate JAK2 on

tyrosine sites Y1007/Y1008 and altering phosphorylation of downstream signal transducer and activator of transcription 3 [15]. Adipocyte-specific *Ptp1b* deletion increases adipocyte size and serum levels of glucose and leptin, without affecting body weight [6]. Muscle-specific and liver-specific deletion of *Ptp1b* has no effect on body weight but, in contrast to adipocyte *Ptp1b* deletion, improves peripheral insulin sensitivity and whole-body glucose homeostasis [2, 14]. Liver-specific *Ptp1b* deletion also decreases serum triacylglycerol levels and lowers lipogenic gene expression in livers of HFD-fed mice (*Srebp1c*, *Srebp1a* and *Fas*). It is thought that this may be due to decreased endoplasmic reticulum (ER) stress response induction observed in these mice [16, 17].

Specifically targeting liver PTP1B appears to be an attractive drug therapy for the treatment of metabolic syndrome, as it not only improves whole-body insulin sensitivity and glucose homeostasis but also decreases lipid deposition in the liver, thus potentially limiting the development of comorbidities such as NAFLD.

Previous studies have examined the beneficial effects of liver PTP1B deficiency in *Alb-Cre* mice, which have hepatocyte *Ptp1b* deletion from birth [2]. The aim of this study was to investigate whether inhibiting liver PTP1B in adult mice with established obesity and insulin resistance could reverse the phenotype and therefore present a novel treatment for insulin resistance and type 2 diabetes. To do this, we used a tamoxifen-dependent Cre recombinase system under the control of the serum albumin promoter to target PTP1B specifically in liver.

Methods

Ethics statement All animal procedures were approved by the University of Aberdeen Ethics Review Committee Board and performed under a project licence approved by the Home Office under the Animals (Scientific Procedures) Act 1986 (PPL60/3951).

Animal studies *Ptp1b*^{fl/fl} mice and *SA-Cre-ER*^{T2} mice expressing *Cre-ER*^{T2} recombinase under the control of the serum albumin promoter were described previously [18, 19]. Tamoxifen treatment of mice efficiently induces Cre-mediated recombination of LoxP flanked (floxed) alleles in hepatocytes but not in other cell types or tissues [19]. *SA-Cre-ER*^{T2} mice, when crossed to *Ptp1b*^{fl/fl} mice, provide a tool to temporally control targeted mutagenesis in hepatocytes. Tamoxifen administration induces a liver-specific deletion of *Ptp1b* (hereafter termed *SA-Ptp1b*^{−/−} mice). DNA extraction and genotyping for the *Ptp1b* floxed allele and the presence of *Cre-ER*^{T2} by PCR were performed as described previously [18]. Mice studied were age-matched littermates, which were generated on a C57BL/6 background. Mice were

housed in groups, unless otherwise stated, and maintained at 22–24°C on a 12 h light/dark cycle with free access to food and water. At weaning (~21 days), mice were placed on a standard 3.4% fat chow pellet diet (Rat and Mouse Breeder and Grower; Special Diets Services, DBM, Bathgate, UK) or an HFD (Adjusted Calories Diet, 55% fat; TD.93075, Harlan Teklad, Madison, WI, USA) and their weight was recorded every 2 weeks. The approximate fatty acid profile of the Adjusted Calories Diet (% total fat) was 28% saturated, 30% *trans*, 28% monounsaturated (*cis*) and 14% polyunsaturated (*cis*), as described previously [20]. For insulin signalling experiments, HFD-fed mice were fasted overnight (16 h) and then injected i.p. with saline (154 mmol/l NaCl) or insulin (10 mU/g body weight) for 10 min. Tissues were dissected immediately after cervical dislocation and frozen in liquid nitrogen.

Tamoxifen administration To prepare tamoxifen, ethanol was added to make a 10 mg/100 µl suspension. Sunflower seed oil was then added to prepare a 10 mg/ml solution. This was heated at 55°C for 30 min. This mixture of tamoxifen, ethanol and sunflower oil was then incorporated into the HFD (55% fat) at 0.7 mg tamoxifen/g food. A control HFD (55% fat) containing ethanol and sunflower oil only was simultaneously administered to a control group. Mice were fed the tamoxifen or control HFD for 28 days.

PTP1B activity assay Tissue lysates were prepared in PTP lysis buffer (130 mmol/l NaCl, 20 mmol/l TRIS (pH 7.5), 5 mmol/l EDTA, 1% Triton X-100 (v/v), 0.5% Nonidet P-40 (v/v)-containing protease inhibitors). PTP1B protein was immunoprecipitated using PTP1B antibody (Millipore, Watford, UK) and protein G-sepharose beads. Beads were resuspended in 60 µl of PTP assay buffer (100 mmol/l HEPES [pH 7.6], 2 mmol/l EDTA, 1 mmol/l dithiothreitol (DTT), 150 mmol/l NaCl, 0.5 mg/ml BSA) containing phosphoregulatory peptide (200 µmol/l). The reaction proceeded for 30 min at 30°C with constant shaking. The concentration of phosphate produced (µmol/l) was then measured by absorbance at 620 nm using Biomol Green Reagent (Enzo Life Sciences, Exeter, UK) and phosphate standards.

Histology Frozen tissues were embedded in optimal cutting temperature compound (OCT) and sectioned by cryostat. Samples were stained in haematoxylin and eosin. Slides were visualised using a Zeiss Axioskop microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) and imaged using AxioVision 4.8 digital image processing software (Carl Zeiss Microscopy).

Serum analysis Serum insulin and leptin (catalogue no. 90080 insulin/90030 leptin; CrystalChem, Downers Grove, IL, USA) were determined by ELISA, following manufacturer's

instructions. TNF-α, IL-6, monocyte chemoattractant protein 1 (MCP-1) and resistin were determined by multiplex ELISA assay (MADKMAG-71 K; Millipore), following manufacturer's instructions. Serum glucose (glucose oxidase, catalogue no. TR1503; Thermo Scientific, Cramlington, UK) and serum triacylglycerol (catalogue no. 17628; Sentinel Diagnostics, Milan, Italy; or catalogue no. TR0100; Sigma, Gillingham, UK) were determined using appropriate kits, following manufacturer's instructions. Serum NEFA were determined using a NEFA C kit (catalogue no. 994-75409E; Wako Chemicals, Richmond, VA, USA). Alanine aminotransferase (ALT) activity was determined using an ALT activity assay kit (catalogue no. K752-100; BioVision, Milpitas, CA, USA) to determine liver function and health. Glucose and insulin concentrations were used to calculate the HOMA of insulin resistance (HOMA-IR), a reliable marker of insulin sensitivity [21], which is defined as: fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5. Assays were measured with a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Liver triacylglycerols Pieces of liver measuring ~100 mg were cut and weighed using analytical scales. PBS 1 ml was added to each tube and homogenised. Samples were centrifuged for 15 s at room temperature. The top layer was resuspended with gentle shaking. The supernatant fraction was transferred (without disturbing the pellet) to new 1.5 ml tubes. Triacylglycerols were then assayed using a kit, following manufacturer's instructions (catalogue no. 17628; Sentinel Diagnostics).

Glycogen determination Two ~20 mg pieces of liver were cut and placed into 2 ml tubes; 500 µl 2 mol/l HCl was added to half of the samples and 500 µl 2 mol/l NaOH (to control for free glucose) was added to the other half. All samples were heated for 2 h at 95°C, following which 500 µl 2 mol/l NaOH was added to each tube containing HCl and 500 µl 2 mol/l HCl was added to each tube containing NaOH for neutralisation. Tubes were vortexed and centrifuged for 1 min. Samples were diluted 1:50 in equal volumes of HCl and NaOH; 10 µl of diluted samples was used for the assay. Hexokinase reagent 150 µl (Sigma) was added to each well and incubated for 10 min at room temperature. The assay was measured at 340 nm with a Spectramax Plus 384 spectrophotometer (Molecular Devices).

Glucose and pyruvate tolerance tests For glucose tolerance tests (GTTs), mice were injected i.p., following a 16 h fast, with glucose 1.5–2 mg/g body weight (1.5 mg for HFD-fed mice and 2 mg for mice on chow diet). For pyruvate tolerance tests (PTTs), mice were injected with 1.5 mg/g pyruvate. Tail blood glucose values were measured using glucometers (AlphaTRAK, Abbott Laboratories, Abbott

Park, IL, USA) immediately before and at 15, 30, 60 and 90 or 120 min after injection.

Immunoblotting Tissue lysates were prepared in radio-immunoprecipitation assay buffer containing fresh sodium orthovanadate and protease inhibitors, as described previously [22]. Proteins were separated by 4–12% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed using antibodies from Cell Signaling Technology (New England Biolabs, Hitchin, UK) (unless stated otherwise) against PTP1B (Millipore), β -actin (Thermo Scientific), SH2 domain-containing protein tyrosine phosphatase 2 (SHP2; Santa Cruz, Dallas, TX, USA), phosphorylated glycogen synthase (p-GS) S641, phosphorylated glycogen synthase kinase 3 (p-GSK-3) α/β S21/S9, p-IR Y1162/63 (Invitrogen), p-IR Y1158 (Invitrogen, Carlsbad, CA, USA), p-Akt/protein kinase B (PKB) S473, phosphorylated extracellular signal-regulated kinase (p-ERK)1/2 mitogen-activated protein kinase (MAPK) T202/Y204, p-Akt/PKB T308, p-FKBP-12- rapamycin associated protein (FRAP)/phosphorylated mammalian target of rapamycin (p-mTOR) S2448 (Santa Cruz), p-S6 ribosomal protein S235/236, phosphorylated eukaryotic translation initiation factor-2 α kinase 3 (p-PERK) T980, phosphorylated eukaryotic initiation factor 2 α (p-eIF2 α) S51, phosphorylated stress-activated protein kinase (p-SAPK)/c-Jun NH₂-terminal kinase (JNK) T183/Y185, ERK2 and total Akt/PKB (Santa Cruz). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies, visualised using enhanced chemiluminescence, and quantified by densitometry scanning with Image J or Bio1D software (PqLab, Fareham, UK).

Gene expression analysis Total RNA was isolated from mouse liver and epididymal adipose tissue using TRI Reagent (Ambion, Warrington, UK), according to the manufacturer's protocol. First-strand cDNA was synthesised from 1 μ g total RNA employing the Bioline Bioscript Pre-amplification System (Bioline, London, UK) and oligo(dT)_{12–18}. Target genes were amplified by real-time RT-PCR using 2 μ l diluted cDNA (1:10; 10 μ l), using GoTaq qPCR Master Mix (Promega, Southampton, UK). The Roche LightCycler 480 System (Roche Diagnostics, Burgess Hill, UK) was used for analysis. Relative gene expression was calculated using the comparative Ct(2^{−ΔΔC_t}) method. A geometric mean of three commonly used reference genes, *Hprt* (encoding hypoxanthine-guanine phosphoribosyltransferase), *18S* (encoding 18S ribosomal RNA) and *Gapdh* (encoding glyceraldehyde 3-phosphate dehydrogenase), was used to normalise data. Geometric means of the relative copy numbers of mouse PCRs were followed by melting curves (70–95°C).

Data analysis Data are expressed as mean $\pm$ SEM, and *n* represents the number of mice or biological replicates.

Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparison post hoc tests, repeated measures two-way ANOVA with Bonferroni multiple comparison post hoc tests, one-tailed *t* tests or two-tailed Student's *t* tests, as appropriate. The critical alpha level (*p*) was set at 0.05; *p*<0.05 was considered statistically significant. GraphPad Prism 5 statistical software was used for analyses (www.graphpad.com/scientific-software/prism/).

Results

Inducible liver-specific PTP1B knockdown improves glucose homeostasis Body weight (Fig. 1) was comparable between HFD-fed *SA-Ptr1b*^{−/−} and HFD-fed *Ptr1b*^{fl/fl} control mice throughout the study, and a decrease in PTP1B levels of ~50% was achieved 4 and 12 weeks after tamoxifen treatment in HFD-fed *SA-Ptr1b*^{−/−} mice (Fig. 1b, c, respectively). PTP1B activity was also ~20% lower in HFD-fed *SA-Ptr1b*^{−/−} mice compared with HFD-fed *Ptr1b*^{fl/fl} control mice (Fig. 1d). Considering that PTP1B activity was ~40% lower in *Alb-Ptr1b*^{−/−} mice (which have *Ptr1b* deletion in the liver from birth) (Fig. 1d), this would be consistent with

Fig. 1 Body weight and PTP1B knockdown/activity. **(a)** Body weight of HFD-fed *SA-Ptr1b*^{−/−} (*n*=8) and HFD-fed *Ptr1b*^{fl/fl} control mice (*n*=9). Experimental design and timings of tamoxifen (tam) treatment are also displayed. **(b)** PTP1B knockdown 4 weeks post-tamoxifen treatment (*n*=4–7). **(c)** PTP1B knockdown 12 weeks post-tamoxifen treatment (*n*=4–7). **(d)** PTP1B activity 12 weeks post-tamoxifen treatment. HFD-fed *Ptr1b*^{fl/fl} control mice (*n*=8), HFD-fed *SA-Ptr1b*^{−/−} mice (*n*=8) and HFD-fed *Alb-Ptr1b*^{−/−} mice (*n*=4). Data are represented as mean $\pm$ SEM. White bars/white circles, *Ptr1b*^{fl/fl}; black bars/black circles, *SA-Ptr1b*^{−/−}; grey bar, *Alb-Ptr1b*^{−/−}. Data were analysed by one-tailed or two-tailed Student's *t* test (**p*<0.05; ***p*<0.01; †*p*=0.054)

50% deletion at the protein level. As expected from other studies, tamoxifen treatment caused ~20% body weight loss in both groups of mice, which returned to pre-tamoxifen levels on HFD (Fig. 1). An outline of the experimental design is shown in Fig. 1. As expected, HFD-fed *SA-Ptp1b*^{-/-} mice displayed no differences in glucose tolerance in comparison with *Ptp1b*^{fl/fl} mice prior to tamoxifen treatment, on a chow or HFD diet (Fig. 2a, b). Importantly, HFD feeding induced glucose intolerance in both groups of mice, as evidenced by an increased AUC in both groups (Fig. 2d). After tamoxifen treatment, which induced PTP1B knockdown in *SA-Ptp1b*^{-/-} mice only (Fig. 1b, c), HFD-fed *SA-Ptp1b*^{-/-} mice displayed a significantly improved response to glucose challenge and a reversal of glucose intolerance to chow diet feeding levels (Fig. 2a, c, d). Furthermore, fed and fasting serum glucose levels were lower in HFD-fed *SA-Ptp1b*^{-/-} mice compared with HFD-fed *Ptp1b*^{fl/fl} control mice 4 and 12 weeks after tamoxifen treatment (Fig. 2f, g). Fed serum insulin levels tended to be lower in *SA-Ptp1b*^{-/-} mice, although this did not reach significance (Fig. 2h). Fasting

serum insulin levels, however, were significantly lower 4 weeks after tamoxifen treatment in HFD-fed *SA-Ptp1b*^{-/-} mice compared with HFD-fed *Ptp1b*^{fl/fl} controls (Fig. 2i). Importantly, HOMA-IR, which represents an index of insulin resistance, was significantly lower in HFD-fed *SA-Ptp1b*^{-/-} mice 4 and 12 weeks after tamoxifen treatment (Fig. 2j).

Inducible liver-specific PTP1B knockdown improves lipid homeostasis Haematoxylin and eosin staining revealed a high level of inter-animal variability when examining lipid deposition in livers of HFD-fed *SA-Ptp1b*^{-/-} and *Ptp1b*^{fl/fl} mice (Fig. 3a). We therefore also assessed total liver triacylglycerol levels, which revealed that they were significantly lower in the livers of HFD-fed *SA-Ptp1b*^{-/-} mice compared with HFD-fed *Ptp1b*^{fl/fl} controls (Fig. 3b). There were no significant differences between groups in ALT activity (Fig. 3c). Fed serum NEFA were significantly decreased 4 and 12 weeks after tamoxifen treatment in HFD-fed *SA-Ptp1b*^{-/-} mice compared with *Ptp1b*^{fl/fl} controls (Fig. 3d). HFD-fed *SA-Ptp1b*^{-/-} mice also displayed

Fig. 2 Inducible liver-specific PTP1B knockdown improves glucose homeostasis. **(a)** GTT of both groups on chow diet prior to tamoxifen (tam) treatment. **(b)** GTT of both groups on HFD for 8 weeks prior to tamoxifen treatment. **(c)** GTT of both groups on HFD 3 weeks after tamoxifen treatment. **(d)** AUC of GTTs. **(e)** PTT of both groups on HFD 12 weeks after tamoxifen treatment. **(f)** Fed serum glucose levels. **(g)** Fasting serum glucose levels. **(h)** Fed serum insulin levels. **(i)** Fasting serum insulin levels. **(j)** HOMA-IR. *SA-Ptp1b*^{-/-} and *Ptp1b*^{fl/fl} control

groups are indicated in the figures. For all experiments $n=8$ for HFD-fed *SA-Ptp1b*^{-/-} mice and $n=9$ for HFD-fed *Ptp1b*^{fl/fl} control mice. Data are represented as mean $\pm$ SEM. White bars/white circles, *Ptp1b*^{fl/fl}; black bars/black circles, *SA-Ptp1b*^{-/-}. Data were analysed by repeated measures two-way ANOVA with Bonferroni multiple comparison post hoc tests, one-way ANOVA with Tukey's multiple comparison post hoc tests or two-tailed Student's *t* test, where appropriate ($p<0.05$)

Fig. 3 Lipid homeostasis is improved with inducible liver-specific PTP1B knockdown. **(a)** Haematoxylin and eosin staining of livers. **(b)** Liver triacylglycerol assay from HFD-fed *SA-Ptp1b*^{-/-} mice and HFD-fed *Ptp1b*^{fl/fl} control mice. **(c)** ALT activity assay. **(d)** Fed serum NEFA assay. **(e)** Fasting serum NEFA assay. **(f)** Fed serum triacylglycerol assay. **(g)** Fasting serum triacylglycerol assay. **(h)** Fed circulating leptin assay. **(i)** Fasting circulating leptin assay. *SA-Ptp1b*^{-/-} and *Ptp1b*^{fl/fl} control groups are indicated in the figures. For all experiments *n*=8 for HFD-fed *SA-Ptp1b*^{-/-} mice and *n*=9 for HFD-fed *Ptp1b*^{fl/fl} control mice. Data are represented as mean ± SEM. White bars, *Ptp1b*^{fl/fl}; black bars, *SA-Ptp1b*^{-/-}. Data were analysed by one-way ANOVA with Tukey's multiple comparison post hoc tests or two-tailed Student's *t* test, where appropriate (**p*<0.05)

significantly lower fasting serum NEFA 12 weeks after tamoxifen treatment (Fig. 3e). Fed and fasting serum triacylglycerol levels were significantly decreased both 4 and 12 weeks after tamoxifen treatment compared with pre-tamoxifen levels in HFD-fed *SA-Ptp1b*^{-/-} mice only (Fig. 3f, g). Moreover, both fed and fasting serum leptin levels were significantly lower after 4 weeks of tamoxifen treatment in HFD-fed *SA-Ptp1b*^{-/-} mice compared with HFD-fed *Ptp1b*^{fl/fl} control mice (Fig. 3h, i). Furthermore, IL-6, MCP-1, resistin and TNF-α were measured in *Ptp1b*^{fl/fl} control and *SA-Ptp1b*^{-/-} mice in the fasted state (Table 1). *SA-Ptp1b*^{-/-} mice displayed lower IL-6 after 12 weeks of tamoxifen treatment compared with *Ptp1b*^{fl/fl} control mice (*p*=0.059; two-tailed *t* test), whilst MCP-1 was increased in *SA-Ptp1b*^{-/-} mice 4 weeks after tamoxifen treatment compared with *Ptp1b*^{fl/fl} control mice. Resistin was not different between groups at any time point (Table 1) and TNF-α concentrations were below the level of detection of the mouse multiplex ELISA (data not shown).

Table 1 Serum glucose levels of IL-6, MCP-1 and resistin before and after 4 and 12 weeks of tamoxifen treatment

Variable	<i>Ptp1b</i> ^{fl/fl} (<i>n</i> =6)	<i>SA-Ptp1b</i> ^{-/-} (<i>n</i> =6)
IL-6 (pmol/l)		
Before treatment	1.04±0.39	0.65±0.18
After 4 weeks	0.31±0.08	0.71±0.32
After 12 weeks	1.43±0.43	0.5±0.07†
MCP-1 (pmol/l)		
Before treatment	1.53±0.1	1.27±0.26
After 4 weeks	0.64±0.26	2.82±0.63*
After 12 weeks	1.17±0.47	1.81±0.52
Resistin (pmol/l)		
Before treatment	108.13±21.47	83.28±15.55
After 4 weeks	102.38±14.18	81.90±22.28
After 12 weeks	77.04±17.38	79.68±13.53

Data are represented as mean ± SEM and were analysed using one-way ANOVA with Tukey's multiple comparison post hoc tests (**p*<0.05; †*p*=0.059)

Inducible liver-specific PTP1B knockdown improves suppression of hepatic gluconeogenesis To assess if most of the improvements in glucose homeostasis were due to improvements in suppression of hepatic gluconeogenesis, we performed a PTT. HFD-fed *SA-Ptp1b*^{-/-} mice displayed a significantly improved response to pyruvate challenge following overnight fasting compared with HFD-fed *Ptp1b*^{fl/fl} controls (Fig. 2e). This is consistent with increased insulin-induced GS dephosphorylation after both 4 and 12 weeks of tamoxifen treatment (Fig. 4a–d). Liver glycogen content was unaltered between *SA-Ptp1b*^{-/-} and *Ptp1b*^{fl/fl} control mice (5.97±3.41 vs 8.32±2.93 µg/mg, respectively). Surprisingly, components of the classical insulin signalling pathway were unchanged with liver PTP1B knockdown both 4 and 12 weeks after tamoxifen treatment (Fig. 4a, b, e). Furthermore, insulin signalling in muscle and epididymal white adipose tissue (WAT) was comparable between *SA-Ptp1b*^{-/-} and *Ptp1b*^{fl/fl} control mice (Fig. 4f, g).

Inducible liver-specific PTP1B knockdown is associated with decreased expression of liver gluconeogenic genes To assess the mechanism(s) behind improvements in whole-

body glucose and lipid homeostasis, we analysed the expression of genes involved in gluconeogenesis and lipogenesis. Consistent with the physiological data from PTTs (Fig. 2e) and signalling data (Fig. 4a–d), HFD-fed *SA-Ptp1b*^{-/-} mice displayed a decrease in gluconeogenic markers in comparison with HFD-fed *Ptp1b*^{fl/fl} control mice, as evidenced by decreased liver gene expression levels of *Pepck* (Table 2). Moreover, *Pparg* was significantly decreased in these mice (Table 2). Furthermore, *Hmgcs1* was increased in HFD-fed *SA-Ptp1b*^{-/-} mice compared with HFD-fed *Ptp1b*^{fl/fl} control mice (Table 1). HFD-fed *SA-Ptp1b*^{-/-} mice exhibited unaltered lipogenic gene expression in liver or epididymal WAT compared with *Ptp1b*^{fl/fl} control mice (Table 1). Lipolytic and adipokine gene expression levels were unaltered between the groups in epididymal WAT (Table 2).

Inducible liver-specific PTP1B knockdown decreases ER stress At the gene expression level, there was a significant decrease in *Grp94* in HFD-fed *SA-Ptp1b*^{-/-} mice compared with HFD-fed *Ptp1b*^{fl/fl} control mice (Table 2). At the protein level, HFD-fed *SA-Ptp1b*^{-/-} mice exhibited significantly

Fig. 4 Inducible liver-specific PTP1B knockdown improves suppression of hepatic gluconeogenesis. **(a)** Liver insulin signalling 4 weeks post-tamoxifen (tam) in HFD-fed *Ptp1b*^{fl/fl} and HFD-fed *SA-Ptp1b*^{-/-} mice after injection with saline or insulin (10 mU/g). **(b)** Liver insulin signalling 12 weeks post-tamoxifen in HFD-fed *Ptp1b*^{fl/fl} and HFD-fed *SA-Ptp1b*^{-/-} mice after injection with saline or insulin (10 mU/g). **(c)** Quantification of GS immunoblot 4 weeks post-tamoxifen: HFD-fed *Ptp1b*^{fl/fl} control mice, *n*=7 (three saline/four insulin); HFD-fed *SA-Ptp1b*^{-/-} mice, *n*=10 (three saline/seven insulin). **(d)** Quantification of GS immunoblot 12 weeks post-tamoxifen: HFD-fed *Ptp1b*^{fl/fl} control mice, *n*=7 (three saline/four insulin); HFD-fed *SA-Ptp1b*^{-/-} mice, *n*=10

(three saline/seven insulin). **(e)** Liver IR phosphorylation by immunoprecipitation 12 weeks post-tamoxifen: HFD-fed *Ptp1b*^{fl/fl} control mice, *n*=6 (two saline/four insulin); HFD-fed *SA-Ptp1b*^{-/-} mice, *n*=6 (two saline/four insulin). **(f)** Muscle insulin signalling 12 weeks post-tamoxifen. **(g)** Epididymal WAT insulin signalling 12 weeks post-tamoxifen: HFD-fed *Ptp1b*^{fl/fl} control mice, *n*=7 (three saline/four insulin); HFD-fed *SA-Ptp1b*^{-/-} mice, *n*=7 (three saline/four insulin). Data are represented as mean ± SEM. White bars, *Ptp1b*^{fl/fl}; black bars, *SA-Ptp1b*^{-/-}. Data were analysed by one-way ANOVA with Tukey's multiple comparison post hoc tests or two-tailed Student's *t* test, where appropriate (**p*<0.05)

Table 2 Lipid and glucose metabolism gene expression in liver and epididymal WAT

Variable	<i>Ptp1b</i> ^{fl/fl} (n=6–7)	<i>SA-Ptp1b</i> ^{-/-} (n=6–8)	<i>p</i> value
Liver metabolism			
<i>Fas</i>	1.0±0.06	1.0±0.23	0.983
<i>Srebp1c</i>	1.0±0.11	1.4±0.24	0.155
<i>Srebp1a</i>	1.0±0.22	1.3±0.22	0.380
<i>Srebp2</i>	1.0±0.20	1.1±0.23	0.751
<i>Hmgcs1</i>	1.0±0.14	1.9±0.32*	0.023
<i>Pparg</i>	1.0±0.12	0.6±0.08*	0.023
<i>Ppara</i>	1.0±0.14	0.8±0.12	0.307
<i>Pgc1a</i>	1.0±0.13	0.7±0.07	0.056
<i>Pepck</i>	1.0±0.14	0.6±0.05*	0.018
<i>G6p</i>	1.0±0.26	0.7±0.15	0.289
Liver ER stress			
<i>Bip</i>	1.0±0.08	0.9±0.08	0.317
<i>Grp94</i>	1.0±0.12	0.6±0.10*	0.043
<i>Chop</i>	1.0±0.05	1.1±0.22	0.667
<i>Xbp Spliced</i>	1.0±0.13	1.3±0.16	0.241
<i>Xbp Total</i>	1.0±0.08	1.2±0.15	0.268
<i>Atf4</i>	1.0±0.06	1.1±0.09	0.296
Adipose tissue metabolism			
<i>Fas</i>	1.0±0.11	0.8±0.14	0.303
<i>Srebp1c</i>	1.0±0.20	1.2±0.21	0.605
<i>Srebp1a</i>	1.0±0.15	1.1±0.13	0.565
<i>Pparg</i>	1.0±0.10	0.9±0.16	0.622
<i>Ppara</i>	1.0±0.27	1.2±0.43	0.775
<i>Hsl</i>	1.0±0.17	0.9±0.13	0.673
<i>Atgl</i>	1.0±0.17	0.7±0.11	0.150
<i>Rbp4</i>	1.0±0.15	0.8±0.19	0.461
<i>Leptin</i>	1.0±0.27	0.9±0.29	0.820
<i>Adipoq</i>	1.0±0.15	0.6±0.20	0.185
<i>Resistin</i>	1.0±0.23	0.9±0.23	0.792
<i>Glut4</i>	1.0±0.17	0.8±0.12	0.315
<i>Pepck</i>	1.0±0.17	0.7±0.19	0.272
<i>F480</i>	1.0±0.44	0.7±0.38	0.652

Data are represented as mean ± SEM and presented as fold change relative to *Ptp1b*^{fl/fl} group; data were analysed using two-tailed Student's *t* test (**p*<0.05)

lower phosphorylation of PERK, eIF2α and JNK2 when compared with *Ptp1b*^{fl/fl} control mice (Fig. 5a–d).

Discussion

There is a growing body of evidence to suggest that PTP1B inhibitors hold great promise for the treatment of type 2 diabetes as well as cancer [3, 4, 8, 16–18, 22–30]. Numerous

Fig. 5 Inducible liver-specific PTP1B knockdown reduces ER stress. (a) Representative blot of liver ER stress signalling 12 weeks post-tamoxifen treatment in HFD-fed *Ptp1b*^{fl/fl} and HFD-fed *SA-Ptp1b*^{-/-} mice. (b–e) Quantification of p-PERK, p-eIF2α, p-JNK2 and p-JNK1 immunoblots: HFD-fed *Ptp1b*^{fl/fl} control mice, n=7; HFD-fed *SA-Ptp1b*^{-/-} mice, n=10. Data are represented as mean ± SEM. White bars, *Ptp1b*^{fl/fl}; black bars, *SA-Ptp1b*^{-/-}. Data were analysed by two-tailed Student's *t* test (**p*<0.05; ****p*<0.001)

mouse and human studies have demonstrated that decreasing PTP1B in various tissues including muscle, liver and brain leads to a multitude of beneficial effects [17, 18, 22, 31]. Liver-specific *Ptp1b* knockout in mice (*Alb-Ptp1b*^{-/-}) led to improved glucose homeostasis and decreased triacylglycerol levels independently of changes in body weight [17]. Previous studies, however, investigated mice with a knockout of *Ptp1b* from birth and have therefore examined the effects of *Ptp1b* deletion in preventing type 2 diabetes but not in treating already obese and insulin-resistant states. Using a tamoxifen-dependent Cre recombinase system, we now demonstrate that decreasing liver PTP1B by ~50% in obese and insulin-resistant adult mice leads to a reversal of glucose intolerance and improvements in lipid homeostasis, and that these effects are manifested within just a matter of weeks after hepatic PTP1B knockdown.

As expected, and reported by others, oral tamoxifen treatment caused a transient decrease in body weight in both groups of mice [32–34]. As with other mouse models of *Ptp1b*-specific deletion [17, 22], body weight of the inducible liver-specific *Ptp1b* knockout mice did not differ from that of control mice. PTP1B knockdown decreased PTP1B protein levels by ~50% and PTP1B activity by ~20% in livers from *SA-Ptp1b*^{-/-} mice. A 50% knockdown is less than was observed previously in livers from *Alb-Ptp1b*^{-/-} mice (achieving ~80% hepatic *Ptp1b* deletion and ~40% activity inhibition) [16, 17]. It has recently been reported that different Cre lines display different degrees of efficiency and specificity [35]. In addition to differences amongst Cre

mice, different floxed gene loci were shown to display a range of sensitivity to recombination when using different Cre lines [35]. However, a 50% decrease in PTP1B levels is physiologically relevant, as PTP1B inhibitors would only be expected to achieve approximately these levels [24].

In agreement with previous studies, glucose homeostasis is improved in *SA-Ptp1b*^{-/-} mice compared with control mice [17]. Interestingly, glucose tolerance of *SA-Ptp1b*^{-/-} mice returned to the responsiveness measured in these mice on a chow diet, suggesting a reversal in glucose intolerance that was caused by 12 weeks of HFD feeding prior to inhibition of hepatic PTP1B. Furthermore, *SA-Ptp1b*^{-/-} mice exhibited significantly lower blood glucose levels in response to a pyruvate bolus, suggesting an increased ability of insulin to suppress hepatic gluconeogenesis. Consistent with these physiological data and our previous studies using *Alb-Ptp1b*^{-/-} mice, we observed increased insulin-induced GS dephosphorylation and decreased expression of the gluconeogenic gene *Pepck* in livers of *SA-Ptp1b*^{-/-} mice, in the absence of changes in liver glycogen content [17]. This suggests that *SA-Ptp1b*^{-/-} mice have an improved gluconeogenic response, efficiently shutting down hepatic glucose production compared with control mice.

It is interesting to note that in our experiment insulin treatment of the control mice led to increased GS phosphorylation, whilst in the *SA-Ptp1b*^{-/-} mice it led to the expected dephosphorylation. At the moment, it is unclear how hepatic PTP1B inhibition affects GS phosphorylation independently of its effects on the IR; however, PTP1B has been shown to regulate protein phosphatase 2 (PP2A) activation [36] as well as regulate hepatic *Srebp1* gene expression through the PP2A axis [37], which may then affect the GS hepatic phosphorylation state [38]. This is currently under investigation in our laboratory, but is consistent with data from *Ptp1b*^{-/-} immortalised cells treated with insulin, which were also shown to exhibit enhanced dephosphorylation of the S641 site on GS [39].

Liver *Ptp1b* deletion has previously been shown to decrease serum triacylglycerol with lower expression of lipogenic genes [17]. This suggests that PTP1B knockdown may be a suitable therapy for NAFLD, which is characterised by increased hepatic lipid accumulation and insulin resistance. A recent study showed that the dietary supplement curcumin inhibits PTP1B and prevents hepatic steatosis in fructose-fed rats, providing support for this notion [40]. Here, we demonstrate that PTP1B knockdown in obese and diabetic mice results in lower liver triacylglycerol levels associated with decreased expression of *Pparg*, which has been found to be elevated in fatty livers [41].

Interestingly, a paradoxical phenotype was previously observed in *Alb-Ptp1b*^{-/-} mice, which displayed increased hepatic insulin signalling and decreased expression levels of hepatic *Srebp1c*, *Fas* and other lipogenic markers [16, 17]. It

is suggested that PTP1B may affect *Srebp1* expression via a non-insulin-signalling pathway in the liver involving effects on PP2A activity [23, 37]. No differences were noted in *Srebp1a*, *Srebp1c* or *Fas* in the current study; it may be that a 50% PTP1B knockdown is not sufficient to measure detectable changes or it may be due to the differences in timing of the *Ptp1b* deletion.

We have previously shown that *Alb-Ptp1b*^{-/-} mice, which delete hepatic *Ptp1b* from birth, are protected against HFD-induced hepatic ER stress [17]. Consistent with this, *SA-Ptp1b*^{-/-} mice also have decreased phosphorylation levels of PERK, eIF2 α and JNK2, indicating that ~50% knockdown of PTP1B can temporally improve ER stress. Moreover, in the absence of changes in the classical IR signalling, this study suggests that improvements in lipid homeostasis observed with hepatic PTP1B knockdown may be due to decreased ER stress response signalling.

SA-Ptp1b^{-/-} mice displayed significantly lower fed and fasting leptin levels 4 weeks after PTP1B knockdown was induced. This is the first time that PTP1B knockdown in the liver has been reported to affect circulating leptin levels. PTP1B has been well documented to regulate leptin receptor signalling [18, 42]. Leptin action in the liver remains, however, inconclusive. Diet-induced obese rats have been shown to exhibit decreased hepatic levels of leptin receptor transcripts [43, 44]. Moreover, leptin treatment of wild-type mice led to increased mRNA expression of several isoforms of the leptin receptor, including the long form of the receptor (Ob-Rb) [45], suggesting that the liver may be an important site of leptin action. Furthermore, overexpression of PTP1B in the liver was shown to restrict the ability of leptin to lower blood glucose levels and suppress food intake [46]. It was suggested that strategies aimed at suppressing PTP1B specifically in the liver could improve both hepatic insulin and leptin sensitivity [46]. Revealing the mechanism(s) behind our current observations should form part of future studies.

Overall, tissue-specific knockout/knockdown studies of PTP1B have revealed key roles for brain, liver and muscle PTP1B in the regulation of global energy and glucose homeostasis. We now demonstrate that liver PTP1B knockdown not only prevents but can also reverse established insulin resistance and glucose intolerance, as well as decrease ER stress and fat accumulation in the liver in obese and insulin-resistant states. Inhibition of PTP1B remains a promising potential therapy for type 2 diabetes treatment as well as a potential protection against the development of NAFLD.

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