Inducible Liver-Specific Knockdown of Protein Tyrosine Phosphatase 1B Improves Glucose and Lipid Homeostasis in Adult Mice

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Abstract word count: 234

Main text word count: 3930

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, 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: To investigate this, 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 within HFD to induce liver-specific deletion of *Ptp1b* (*SA-Ptp1b*$^{-/-}$ mice). Body weight, glucose homeostasis, lipid homeostasis, serum adipokines, insulin signalling and 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 fasted blood glucose and insulin levels, lower HOMA-IR, circulating leptin, serum and liver triglycerides, serum free fatty acids and decreased HFD-induced ER stress. This was associated with decreased glycogen synthase, PERK, eIF2α and JNK2 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 subjects that are already obese/insulin resistant may have relatively rapid, beneficial therapeutic effects.

KEY WORDS: Liver, PTP1B, Phosphatase, NAFLD, Disease, Glucose, Lipid, Insulin, Leptin, ER Stress.

Abbreviations

ER	Endoplasmic reticulum
GTT	Glucose tolerance test
HFD	High-fat diet
HOMA-IR	Homeostatic model assessment of insulin resistance
IR	Insulin receptor
JAK2	Janus kinase 2
NAFLD	Non-alcoholic fatty liver disease
PTP1B	Protein tyrosine phosphatase 1B

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 dyslipidemia [3] and non-alcoholic fatty liver disease (NAFLD), which is the most common liver disease across 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 phosphatise 1B (PTP1B) 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 high-fat diet (HFD) [8, 9]. PTP1B antisense oligonucleotides were shown to effectively lower PTP1B levels in liver and fat, enhance insulin signalling, as well as decrease adiposity in *ob/ob* and *db/db* mice [10, 11]. Lipogenic genes were down-regulated in fat and liver, including diminished *Pparγ* gene expression in adipose tissue [12]. However, whether these positive effects were due to loss of PTP1B in the liver and/or adipose tissue or any other tissue(s) was 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].

Neuronal *Ptp1b*$^{-/-}$ mice display decreased body mass, reduced food intake and enhanced energy expenditure on a high-fat diet (HFD) [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 downstream STAT3 phosphorylation [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 triglyceride levels and lowers lipogenic gene expression in livers of mice fed HFD (*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 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 co-morbidities such as NAFLD.

Previous studies have examined beneficial effects of liver PTP1B deficiency using *Alb-Cre* mice which induces hepatocyte-*Ptp1b* deletion from birth [2]. The aim of this study was to investigate whether inhibiting liver-PTP1B in adult mice with already 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.

Materials and Methods

Ethics statement. All animal procedures were approved by the University of Aberdeen Ethics Review Committee Board and performed under a project license 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*$^{-/-}$). 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 22-24ºC on a 12-h light/dark cycle with free access to food and water. At weaning (~21 days), mice were placed on standard 3.4% fat chow pellet diet (Rat and Mouse Breeder and Grower, Special Diets Services, DBM, Scotland) or HFD (Adjusted Calories Diet, 55 % fat, Harlan

Teklad, USA) and weight was recorded every two weeks. The approximate fatty acid profile of 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 hours) and then injected intraperitoneally with saline or insulin (10 mU/g body weight) for 10 minutes. Tissues were dissected immediately post-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 minutes. This mixture of tamoxifen, ethanol and sunflower oil was then incorporated into HFD (55% fat) at 0.7 mg tamoxifen/gram of food. A control HFD (55% fat) was simultaneously administered to a control group containing ethanol and sunflower oil only. 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) and protein G-sepharose beads. Beads were re-suspended in 60 µl of PTP assay buffer (100 mmol/l Hepes (pH 7.6), 2 mmol/l EDTA, 1 mmol/l DTT, 150 mmol/l NaCl, 0.5 mg/ml BSA) containing phosphoregulatory peptide (200 µmol/l). The reaction proceeded for 30 minutes 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) and phosphate standards.

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

Serum analysis. Serum insulin and leptin (CrystalChem, Downers Grove, USA, Cat 90080 Insulin/90030 Leptin) were determined by ELISA, following manufacturer's instructions. TNFα, IL-6, MCP-1 and Resistin were determined by multiplex ELISA assay (MADKMAG-71K, Millipore), following manufacturer's instructions. Serum glucose (glucose oxidase, Thermo Scientific, Cat TR1503) and serum triglycerides (Sentinel Diagnostics, Milan, Italy, Cat 17628 or Sigma, Cat TR0100) were determined using appropriate kits, following manufacturer's instructions. Serum free fatty acids were determined using a non-esterified fatty acid (NEFA C) kit (Wako Chemicals, Virginia, USA, Cat 994-75409E). Alanine aminotransferase activity was determined using an alanine aminotransferase activity assay kit (BioVision, California, USA, Cat K752-100) to determine liver function and health. Glucose and insulin concentrations were used to calculate the homeostasis model assessment of insulin resistance (HOMA-IR), a reliable marker of insulin sensitivity [21], which is defined as: fasting glucose (mmol/l) X fasting insulin (mU/l)/22.5. Assays were measured with a Spectramax Plus 384 spectrophotometer (Molecular Devices, CA, USA).

Liver triglycerides. ~100 mg pieces of liver were cut and weighed using analytical scales. 1 ml PBS was added to each tube and homogenised. Samples were centrifuged for 15 seconds at room temperature. The top layer was resuspended with gentle shaking. The supernatant was transferred (without disturbing the pellet) to new 1.5 ml tubes. Triglycerides were then assayed using a kit, following manufacturer's instructions (Sentinel Diagnostics, Milan, Italy, Cat 17628).

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. 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 neutralization. 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 were used for the assay. 150 µl of hexokinase reagent (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, CA, USA).

Glucose and pyruvate tolerance tests. For glucose tolerance tests, mice were intraperitoneally injected, following a 16-hour fast, with 1.5-2 mg/g (1.5 mg for HFD-fed and 2 mg for mice on chow diet) body weight glucose. For pyruvate tolerance tests, mice were injected with 1.5 mg/g pyruvate. Tail blood glucose values were measured using glucometers (AlphaTRAK, Berkshire, UK) immediately before and at 15, 30, 60, and 90 or 120 min post-injection.

Immunoblotting. Tissue lysates were prepared in RIPA 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 (Cell Signaling by NEB, Hitchin, UK) (unless stated otherwise) against PTP1B (Millipore), Beta-Actin (Thermo Scientific), SHP2 (Santa Cruz), pGS S641, pGSK3α/β S21/S9, pIR Y1162/63 (Invitrogen), pIR Y1158 (Invitrogen), pAkt/PKB S473, pERK1/2 MAPK T202/Y204, pAkt/PKB T308, p-FRAP/p-mTOR S2448 (Santa Cruz), pS6 ribosomal protein S235/236, pPERK T980, peIF2α S51, pSAPK/JNK T183/Y185, ERK2 and total Akt/PKB (Santa Cruz). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies, visualized using enhanced chemiluminescence, and quantified by densitometry scanning with Image J or Bio1D software (PeqLab, 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 synthesized from 1 µg of total RNA employing the Bioline Bioscript™ Pre-amplification System and oligo(dT)$_{12-18}$. Two (2) µl of diluted cDNA (1:10) was used to amplify target genes by real-time RT-PCR (10 µl), 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−ΔΔCt) method. A geometric mean of three commonly used reference genes; hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), 18S ribosomal RNA (*18S*) and Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used to normalise data. A geometric mean of the relative copy numbers of mouse PCRs were followed by melting curves (70-95°C).

Data analysis. Data are expressed as mean ± 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-tests, repeated measures two-way ANOVA with Bonferroni multiple comparisons post-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.

Results

Inducible liver-specific *Ptp1b* knockdown improves glucose homeostasis. Body weight (Figure 1) was comparable between HFD-fed *SA-Ptp1b*$^{-/-}$ and HFD-fed *Ptp1b*$^{fl/fl}$ control mice throughout the study and ~50% decrease in PTP1B levels was achieved at 4- and 12-weeks post-tamoxifen treatment in HFD-fed *SA-Ptp1b*$^{-/-}$ mice (Figure 1b and c, respectively). PTP1B activity was also ~20% lower in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with HFD-fed *Ptp1b*$^{fl/fl}$ control mice (Figure 1d). Considering that PTP1B activity was ~40% lower in *Alb-Ptp1b*$^{-/-}$ mice (which have *Ptp1b* deletion in the liver from birth) (Figure 1d), this would be

consistent with 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 (Figure 1). An outline of the experimental design is shown in Figure 1. As expected, HFD-fed *SA-Ptp1b*$^{-/-}$ mice displayed no differences in glucose tolerance in comparison to *Ptp1b*$^{fl/fl}$ mice prior to tamoxifen treatment, on chow or HFD (Figure 2a and b). Importantly, HFD-feeding induced glucose intolerance in both groups of mice, as evidenced by increased area under the curve in both groups (Figure 2d). After tamoxifen treatment, which induced PTP1B knockdown in *SA-Ptp1b*$^{-/-}$ mice only (Figure 1b and 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 (Figure 2a, c and d). Furthermore, fed and fasted serum glucose levels were lower in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with HFD-fed *Ptp1b*$^{fl/fl}$ control mice, at 4- and 12-weeks post-tamoxifen treatment (Figure 2f and g). Fed serum insulin levels tended to be lower in *SA-Ptp1b*$^{-/-}$ mice, although this did not reach significance (Figure 2h). However, fasting serum insulin levels were significantly lower at 4-weeks post-tamoxifen treatment in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with HFD-fed *Ptp1b*$^{fl/fl}$ controls (Figure 2i). Importantly, HOMA-IR, which represents an index of insulin resistance, was significantly lower in HFD-fed *SA-Ptp1b*$^{-/-}$ mice, at 4- and 12-weeks post-tamoxifen treatment (Figure 2j).

Inducible liver-specific *Ptp1b* knockdown improves lipid homeostasis. Hematoxylin 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 (Figure 3a). We therefore also assessed total liver triglyceride levels, which revealed that liver triglycerides were significantly lower in the livers of HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with HFD-fed *Ptp1b*$^{fl/fl}$ controls (Figure 3b). There were no significant differences between groups in alanine aminotransferase activity (Figure 3c). Fed serum free-fatty acids were significantly

decreased at 4- and 12-weeks post-tamoxifen treatment in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with *Ptp1b*$^{fl/fl}$ controls (Figure 3d). HFD-fed *SA-Ptp1b*$^{-/-}$ mice also displayed significantly lower fasting serum free-fatty acids at 12-weeks post-tamoxifen treatment (Figure 3e). Fed and fasted serum triglycerides were significantly decreased at both 4- and 12-week post-tamoxifen treatment compared with the starting pre-tamoxifen levels in HFD-fed *SA-Ptp1b*$^{-/-}$ mice only (Figure 3f and g). Moreover, both fed and fasted serum leptin levels were significantly lower at 4 weeks post-tamoxifen treatment in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with HFD-fed *Ptp1b*$^{fl/fl}$ control mice (Figure 3h and i). Furthermore, IL-6, MCP-1, Resistin and TNFα were measured in *Ptp1b*$^{fl/fl}$ control and *SA-Ptp1b*$^{-/-}$ mice in the fasted state (Table 2). *SA-Ptp1b*$^{-/-}$ mice displayed lower IL-6 at 12-weeks post-tamoxifen compared with *Ptp1b*$^{fl/fl}$ control mice (P = 0.059; two-tailed t-test), whilst MCP-1 was increased in *SA-Ptp1b*$^{-/-}$ mice at 4-weeks post-tamoxifen compared with *Ptp1b*$^{fl/fl}$ control mice. Resistin was not different between groups at any time point (Table 2) and TNFα concentrations were below the level of detection of the mouse multiplex ELISA (data not shown).

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 pyruvate tolerance test. HFD-fed *SA-Ptp1b*$^{-/-}$ mice displayed a significantly improved response to pyruvate challenge following overnight fasting compared with HFD-fed *Ptp1b*$^{fl/fl}$ controls (Figure 2e). This is consistent with increased insulin-induced dephosphorylation of glycogen synthase at both 4- and 12-weeks post-tamoxifen treatment (Figure 4 a, b, c and 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 at either 4- or 12-week post-tamoxifen

(Figure 4a, b and e). Furthermore, insulin signalling in muscle and epididymal WAT was comparable between *SA-Ptp1b*$^{-/-}$ and *Ptp1b*$^{fl/fl}$ control mice (Figure 4f and 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 expression of genes involved in gluconeogenesis and lipogenesis. Consistent with the physiological data from pyruvate tolerance tests (Figure 2e) and signalling data (Figure 4a, b, c and d), HFD-fed *SA-Ptp1b*$^{-/-}$ mice displayed a decrease in gluconeogenic markers in comparison to HFD-fed *Ptp1b*$^{fl/fl}$ control mice, as evidenced by decreased liver gene expression levels of *Pepck* (Table 1). Moreover, *Ppary* was significantly decreased in these mice (Table 1). Furthermore, *Hmgcs1* was increased in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared to HFD-fed *Ptp1b*$^{fl/fl}$ control mice (Table 1). HFD-fed *SA-Ptp1b*$^{-/-}$ mice exhibited unaltered lipogenic gene expression in liver or epididymal white adipose tissue compared to *Ptp1b*$^{fl/fl}$ control mice (Table 1). Lipolytic and adipokine gene expression levels were unaltered between the groups in epididymal white adipose tissue (Table 1).

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 to HFD-fed *Ptp1b*$^{fl/fl}$ control mice (Table 1). At the protein level, HFD-fed *SA-Ptp1b*$^{-/-}$ mice exhibited significantly lower phosphorylation of PERK, eIF2α and JNK2 when compared to *Ptp1b*$^{fl/fl}$ control mice (Figure 5a, b, c and d).

Discussion

There is a growing body of evidence to suggest that PTP1B inhibitors hold great promise for treatment of type 2 diabetes as well as cancer [3, 4, 8, 16-18, 22-30]. Numerous mouse and human studies have demonstrated that decreasing PTP1B in various tissues including muscle,

liver and the 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 levels of triglycerides independent of changes in body weight [17]. However, previous studies investigated mice with a knockout of *Ptp1b* from birth and have therefore examined the effects of *Ptp1b* deletion as a preventative of type 2 diabetes, not as a treatment in the 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 post 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 control mice. *Ptp1b*-knockdown decreased PTP1B protein levels by ~50% and PTP1B activity by ~20% in livers from *SA-Ptp1b*$^{-/-}$ mice. 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 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 dephosphorylation of glycogen synthase 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 to control mice.

It is interesting to note that in our experiment insulin treatment of the control mice led to increased phosphorylation of GS, 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 insulin receptor; however, PTP1B has been shown to regulate PP2A activation [36] as well as regulate hepatic *Srebp1* gene expression through the PP2A axis [37], which may then affect GS hepatic phosphorylation state [38]. This is currently under investigation in our lab, 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 triglyceride 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 behind this notion [40]. Here, we demonstrate that *Ptp1b* knockdown in obese and diabetic mice results

in lower liver triglyceride levels associated with decreased expression of *Pparγ*, which has been found to be elevated in fatty livers [41].

Interestingly, a paradoxical phenotype was previously observed in *Alb-Ptp1b*$^{-/-}$ mice; they 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* gene 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 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, eIF2α 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 fasted 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]. However, leptin action in the liver remains 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 (ObRb) [45], suggesting that the liver may be an important site of leptin action. Furthermore, over-expression 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 does not only prevent, but can reverse established insulin resistance and glucose intolerance and also decrease ER stress and fat accumulation in the liver in the 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.

Acknowledgements

We would like to thank Pierre Chambon and Daniel Metzger (Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, CU. de Strasbourg, France) for kindly providing SA-CRE-ER^{T2} mice. We would like to thank Benjamin Neel (Campbell Family Cancer Research Institute, Ontario Cancer Institute, University of Toronto) and Barbara Kahn (Division of Endocrinology, Diabetes and Metabolism, Beth Israel Deaconess Medical Centre, Boston) for *Ptp1b* floxed mice. We would also like to thank George Mcilroy (Institute of Medical Sciences, School of Medical Sciences, University of Aberdeen) for helping out with glucose tolerance tests.

Funding

This work was supported by the British Heart Foundation project grant awarded to MD (PG/09/048/27675 and PG/11/8/28703) and the Diabetes UK project grant awarded to MD

(BDA/RD08/0003597). CO and EKL are recipients of BBSRC postgraduate studentships. NM is the recipient of a British Heart Foundation intermediate basic research fellowship.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement

CO, EKL, LG, NM, and MD contributed to acquisition of the data. CO, EKL, LG, NM and MD performed the analyses. CO and EKL wrote the first draft of the paper, and LG, DJZ, NM, KKB and MD contributed to the interpretation of data and critical revision of the manuscript. All authors were involved in the writing of the manuscript and approved the final version of the article.

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532

533 Table 1. Lipid and glucose metabolism gene expression in liver and epididymal WAT.

Parameter	*Ptp1b*$^{fl/fl}$ (*n* = 6-7)	*SA-Ptp1b*$^{-/-}$ (*n* = 6-8)	*P* value
Liver metabolism			
Fas	1.0 ± 0.06	1.0 ± 0.23	0.983
Srebp1c	1.0 ± 0.11	1.4 ± 0.24	0.155
Srebp1a	1.0 ± 0.22	1.3 ± 0.22	0.380
Srebp2	1.0 ± 0.20	1.1 ± 0.23	0.751
Hmgcs1	1.0 ± 0.14	1.9 ± 0.32*	0.023
Pparγ	1.0 ± 0.12	0.6 ± 0.08*	0.023
Pparα	1.0 ± 0.14	0.8 ± 0.12	0.307
Pgc1α	1.0 ± 0.13	0.7 ± 0.07	0.056
Pepck	1.0 ± 0.14	0.6 ± 0.05*	0.018
G6p	1.0 ± 0.26	0.7 ± 0.15	0.289
Liver ER stress			
Bip	1.0 ± 0.08	0.9 ± 0.08	0.317
Grp94	1.0 ± 0.12	0.6 ± 0.10*	0.043
Chop	1.0 ± 0.05	1.1 ± 0.22	0.667
Xbp Spliced	1.0 ± 0.13	1.3 ± 0.16	0.241
Xbp Total	1.0 ± 0.08	1.2 ± 0.15	0.268
Atf4	1.0 ± 0.06	1.1 ± 0.09	0.296
Adipose tissue metabolism			
Fas	1.0 ± 0.11	0.8 ± 0.14	0.303
Srebp1c	1.0 ± 0.20	1.2 ± 0.21	0.605
Srebp1a	1.0 ± 0.15	1.1 ± 0.13	0.565
Pparγ	1.0 ± 0.10	0.9 ± 0.16	0.622
Pparα	1.0 ± 0.27	1.2 ± 0.43	0.775
Hsl	1.0 ± 0.17	0.9 ± 0.13	0.673
Atgl	1.0 ± 0.17	0.7 ± 0.11	0.150
Rbp4	1.0 ± 0.15	0.8 ± 0.19	0.461
Leptin	1.0 ± 0.27	0.9 ± 0.29	0.820
Adipoq	1.0 ± 0.15	0.6 ± 0.20	0.185
Resistin	1.0 ± 0.23	0.9 ± 0.23	0.792
Glut4	1.0 ± 0.17	0.8 ± 0.12	0.315
Pepck	1.0 ± 0.17	0.7 ± 0.19	0.272
F480	1.0 ± 0.44	0.7 ± 0.38	0.652

534 Data are presented as fold change relative to *Ptp1b*$^{fl/fl}$ group. Data represented as mean ±
535 SEM. Data were analyzed using two-tailed Student's t test (*$P < 0.05$).

Table 2. Serum glucose parameters IL-6, MCP-1 and Resistin.

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

Data represented as mean ± SEM. Data were analyzed using one-way ANOVA with Tukey's multiple comparison post-tests (*$P < 0.05$; † represents $P = 0.059$).

FIGURE LEGENDS

Figure 1. **Body weight and *Ptp1b* knockdown/activity.** *a*: Body weight of HFD-fed *SA-Ptp1b*$^{-/-}$ (*n* = 8) and HFD-fed *Ptp1b*$^{fl/fl}$ control mice (*n* = 9). Experimental design and timings of tamoxifen treatment also displayed. *b*: PTP1B knockdown 4-weeks post-tamoxifen (*n* = 4-7). *c*: PTP1B knockdown 12-weeks post-tamoxifen (*n* = 4-7). *d*: PTP1B activity 12-weeks post-tamoxifen. HFD-fed *Ptp1b*$^{fl/fl}$ control mice *n* = 8, HFD-fed *SA-Ptp1b*$^{-/-}$ mice *n* = 8 and HFD-fed *Alb-Ptp1b*$^{-/-}$ mice *n* = 4. Data are represented as mean ± SEM. White bars/circles, *Ptp1b*$^{fl/fl}$; black bars/circles *SA-Ptp1b*$^{-/-}$; grey bars *Alb-Ptp1b*$^{-/-}$. Data were analyzed by one-tailed or two-tailed Student's t test (*$P < 0.05$; **$P < 0.01$; † represents $P = 0.054$).

Figure 2. **Inducible liver-specific *Ptp1b* knockdown improves glucose homeostasis.** *a*: Glucose tolerance test (GTT) of both groups on chow diet prior to tamoxifen treatment. *b*:

GTT of both groups on HFD for 8 weeks prior to tamoxifen treatment. *c*: GTT of both groups on HFD at 3 weeks after tamoxifen treatment. *d*: Area under the curve of GTT's. *e*: Pyruvate tolerance test of both groups on HFD 12 weeks after tamoxifen treatment. *f*: Fed serum glucose levels. *g*: Fasted serum glucose levels. *h*: Fed serum insulin levels. *i*: Fasted 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 ± SEM. White bars/circles, *Ptp1b*$^{fl/fl}$; black bars/circles *SA-Ptp1b*$^{-/-}$. Data were analyzed by repeated measures two-way ANOVA with Bonferroni multiple comparisons post-tests, one-way ANOVA with Tukey's multiple comparison post-tests or two-tailed Student's t test, where appropriate (*$P < 0.05$).

Figure 3. **Lipid homeostasis is improved with inducible liver-specific *Ptp1b* knockdown.** *a*: Hematoxylin and eosin staining of livers *b*: Liver triglyceride assay from HFD-fed *SA-Ptp1b*$^{-/-}$ mice (SA) and HFD-fed *Ptp1b*$^{fl/fl}$ control mice (FL). *c*: Alanine aminotransferase activity assay. *d*: Fed serum free fatty acid assay. *e*: Fasted serum free fatty acid assay. *f*: Fed serum triglyceride assay. *g*: Fasted serum triglyceride assay. *h*: Fed circulating leptin assay. *i*: Fasted 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 analyzed by one-way ANOVA with Tukey's multiple comparison post-tests or two-tailed Student's t test, where appropriate (*$P < 0.05$).

Figure 4. **Inducible liver-specific *Ptp1b* knockdown improves suppression of hepatic gluconeogenesis.** *a*: Liver insulin signalling 4-weeks post-tamoxifen 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 glycogen synthase immunoblot 4-weeks post-tamoxifen. HFD-fed *Ptp1b*$^{fl/fl}$ control mice $n = 7$ (3 saline/4 insulin) and HFD-fed *SA-Ptp1b*$^{-/-}$ $n = 10$ (3 saline/7 insulin). *d*: Quantification of glycogen synthase immunoblot 12-weeks post-tamoxifen. HFD-fed *Ptp1b*$^{-fl/fl}$ control mice $n = 7$ (3 saline/4 insulin) and HFD-fed *SA-Ptp1b*$^{-/-}$ $n = 10$ (3 saline/7 insulin). *e*: Liver IR phosphorylation by immunoprecipitation 12-weeks post-tamoxifen. HFD-fed *Ptp1b*$^{fl/fl}$ control mice $n = 6$ (2 saline/4 insulin) and HFD-fed *SA-Ptp1b*$^{-/-}$ $n = 6$ (2 saline/4 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 $n = 7$ (3 saline/4 insulin) and HFD-fed *SA-Ptp1b*$^{-/-}$ $n = 7$ (3 saline/4 insulin). Data are represented as mean ± SEM. White bars, *Ptp1b*$^{fl/fl}$; black bars *SA-Ptp1b*$^{-/-}$. Data were analyzed by one-way ANOVA with Tukey's multiple comparison post-tests or two-tailed Student's t test, where appropriate ($*P < 0.05$).

Figure 5. **Inducible liver-specific *Ptp1b* knockdown reduces ER stress.** *a*: Representative blot of liver ER stress signalling 12-weeks post-tamoxifen in HFD-fed *Ptp1b*$^{fl/fl}$ and HFD-fed *SA-Ptp1b*$^{-/-}$ mice. *b-e*: Quantification of pPERK, peIF2α, pJNK2 and pJNK1 immunoblots. HFD-fed *Ptp1b*$^{fl/fl}$ control mice $n = 7$ and HFD-fed *SA-Ptp1b*$^{-/-}$ $n = 10$. Data are represented as mean ± SEM. White bars, *Ptp1b*$^{fl/fl}$; black bars *SA-Ptp1b*$^{-/-}$. Data were analyzed by two-tailed Student's t test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).