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Protein-tyrosine Phosphatase 1B Expression Is Induced by Inflammation *in Vivo**

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Protein-tyrosine phosphatase 1B (PTP1B) is a major negative regulator of insulin and leptin sensitivity. PTP1B overexpression in adipose tissue and skeletal muscle of humans and rodents may contribute to insulin resistance and obesity. The mechanisms mediating PTP1B overexpression in obese and diabetic states have been unclear. We find that adipose tissue inflammation and the pro-inflammatory cytokine tumor necrosis factor α (TNFα) regulate PTP1B expression *in vivo*. High fat feeding of mice increased PTP1B expression 1.5- to 7-fold in adipose tissue, liver, skeletal muscle, and arcuate nucleus of hypothalamus. PTP1B overexpression in high fat-fed mice coincided with increased adipose tissue expression of the macrophage marker CD68 and TNFα, which is implicated in causing obesity-induced insulin resistance. TNFα increased PTP1B mRNA and protein levels by 2- to 5-fold in a dose- and time-dependent manner in adipocyte and hepatocyte cell lines. TNFα administration in mice increased PTP1B mRNA 1.4- to 4-fold in adipose tissue, liver, skeletal muscle, and hypothalamic arcuate nucleus and PTP1B protein 2-fold in liver. Actinomycin D treatment blocked, and high dose salicylate treatment inhibited by 80%, TNFα-induced PTP1B expression in adipocyte cell lines, suggesting TNFα may induce PTP1B transcription via nuclear factor κB (NFκB) activation. Chromatin immunoprecipitation from adipocyte cell lines and liver of mice demonstrated TNFα-induced recruitment of NFκB subunit p65 to the *PTP1B* promoter *in vitro* and *in vivo*. In mice with diet-induced obesity, *TNFα* deficiency also partly blocked PTP1B overexpression in adipose tissue. Our data suggest that PTP1B overexpression in multiple tissues in obesity is regulated by inflammation and that PTP1B may be a target of anti-inflammatory therapies.

According to current World Health Organization estimates, twice as many people worldwide suffer ill health effects from the accumulation of excess adipose mass (1.6 billion) than from malnutrition (800 million). Obesity contributes to the pathogenesis of many important human diseases, including type 2 diabetes and cancer (1). Obesity is accompanied by resistance to insulin and leptin, key hormones regulating glucose homeostasis and body weight (2). The molecular mechanisms underlying leptin and insulin resistance in obesity are not completely understood.

PTP1B[2] is a major negative regulator of insulin and leptin sensitivity, acting to dephosphorylate the insulin receptor and the leptin receptor-associated Janus kinase 2 (3, 4). PTP1B may also dephosphorylate more distal components of these signaling pathways, such as insulin receptor substrate 1 (5, 6). *In vivo*, PTP1B is widely expressed in multiple cell types and tissues, including skeletal muscle, liver, adipose tissue, and brain (3, 4). *PTP1B* deficiency enhances insulin signaling and sensitivity in skeletal muscle and liver (7–10). *PTP1B*$^{-/-}$ mice also have reduced adiposity and are protected from diet-induced obesity (7, 8) due to enhanced leptin action (11, 12) in neurons (9). Conversely, low level PTP1B overexpression in muscle of transgenic mice causes impaired insulin signaling in muscle and whole body insulin resistance (13). Similarly, PTP1B re-expression in liver of *PTP1B*-deficient mice leads to a marked attenuation of their enhanced insulin sensitivity (14). In humans, *PTP1B* polymorphisms are associated with insulin resistance, obesity, or other characteristics of the metabolic syndrome in some populations (3, 15–17).

Reports of PTP1B overexpression in tissues of insulin-resistant, obese, and/or diabetic animals and humans are somewhat inconsistent. Several studies have reported that PTP1B levels and activity are increased in muscle and adipose tissue of obese, insulin-resistant, and/or diabetic rodents (18–22) and humans (23–26). Increased PTP1B expression in liver has also been reported in some insulin-resistant, obese, or diabetic animal models (19, 21, 22, 27, 28). Other work contradicts these conclusions, showing that PTP1B expression levels are unchanged or even lower than normal in obese and/or diabetic animals (29) and humans (24, 30, 31). In most studies, increased PTP1B expression in obese states correlates with increased PTP1B activity (20, 21, 27), implicating regulation of PTP1B protein expression as a major mechanism mediating increased PTP1B activity. Tyrosine phosphorylation, serine phosphorylation,

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[2] The abbreviations used are: PTP1B, protein-tyrosine phosphatase 1B; TNFα, tumor necrosis factor-α; DEXA, dual-energy x-ray absorptometry; HPRT, hypoxanthine guanine phosphoribosyltransferase; ERK, extracellular signal-regulated kinase; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; ANOVA, analysis of variance; DIO, diet-induced obesity; WAT, white adipose tissue; NFκB, nuclear factor κB.

oxidation, and sumoylation have been reported to regulate PTP1B activity (4, 32), although the roles of these modifications in regulating PTP1B activity *in vivo* are not well understood. It also has been unclear how PTP1B expression is regulated *in vivo*.

Accumulating evidence indicates that obesity is an inflammatory state, with elevation of the pro-inflammatory cytokines TNFα, interleukin-1, and interleukin-6 in adipose tissue or sera (33–35). These cytokines are implicated in the pathogenesis of insulin resistance and may represent a causal link between obesity and diabetes (33, 34). Obesity-associated inflammation appears to be triggered by, and primarily involves, adipose tissue (36, 37). In humans and rodents, obesity-associated inflammation is evidenced by macrophage infiltration of adipose tissue, with macrophages constituting up to 50% of the cells in fat pads of obese rodents (36, 37). Adipose tissue is the source of increased levels of TNFα in obesity (38). Although macrophages are potent sources of pro-inflammatory cytokines, purified populations of isolated adipocytes have been shown to secrete TNFα (35, 38). The triggers of adipose tissue inflammation are not fully understood, but may involve cytokine or chemokine secretion by adipocytes or endothelial cells as adipocyte hypertrophy and necrosis occurs (39).

TNFα inhibits insulin action *in vitro* and *in vivo* by altering expression or activity of multiple proteins in the insulin-signaling pathway in cells (35, 38, 40). Importantly, TNFα treatment decreases insulin-stimulated insulin receptor and insulin receptor substrate tyrosine phosphorylation in cultured cells and tissues (40–42). The TNFα receptors TNFR1 (p55) and TNFR2 (p75) mediate the biological responses to TNFα and are expressed ubiquitously on cells (41, 43). Presently it is unclear whether TNFα is an endocrine or mainly paracrine mediator of insulin resistance in obesity (35).

In the present study, we sought to identify factors that mediate PTP1B overexpression in insulin resistance, obesity, and diabetes. Our data confirm that PTP1B overexpression occurs in insulin-target tissues of several, but not all insulin-resistant, obese, and/or diabetic animal models, suggesting that factors other than insulin resistance, obesity, and diabetes regulate tissue PTP1B overexpression *in vivo*. Importantly, in mice with high fat diet-induced obesity, PTP1B overexpression in liver, adipose tissue, and muscle, as well as the arcuate nucleus of hypothalamus, coincides with increased expression of adipose tissue inflammatory markers and TNFα. TNFα treatment itself is sufficient to increase PTP1B mRNA and protein levels in cultured cells and insulin- and leptin-target tissues of mice, and *TNFα* deficiency partly blocks diet-induced PTP1B overexpression in adipose tissue. Inhibition of transcription or NFκB activation and chromatin immunoprecipitation from cells or tissues suggest TNFα induces PTP1B expression in part by transactivation by NFκB. Our data strongly suggest that the development of inflammation may be a unifying theme underlying tissue PTP1B overexpression in obesity and diabetes.

EXPERIMENTAL PROCEDURES

Animals—Mice and rats were housed at 22 °C with a 12-h/12-h or 14-h/10-h light/dark cycle and fed *ad libitum*. Results are reported from random-fed mice on a standard rodent chow diet unless otherwise indicated. FVB mice were obtained from Taconic (Hudson, NY). Obese leptin-deficient mice (*ob*/*ob*) and lean littermate controls (+/+ or +/*ob*) were obtained from The Jackson Laboratory (Bar Harbor, ME). Obese, diabetic long form leptin receptor-deficient mice (*db*/*db*) and lean littermate controls (+/+ or +/*db*) with the Ks+ strain background were obtained from The Jackson Laboratory. Obese leptin-receptor mutant Zucker rats (*fa*/*fa*) and lean littermate controls (+/+ or +/*fa*) were obtained from Harlan (Indianapolis, IN). Fed and 16-h fasted *ob*/*ob* and *fa*/*fa* and 16-h fasted *db*/*db* mice were examined with their respective lean controls. Homozygous *TNFα*-deficient ($TNF\alpha^{-/-}$) mice with a mixed genetic background of C57BL/6J and 129SvJ strains and wild-type F1 progeny of a C57BL/6J and 129SvJ mating were obtained from The Jackson Laboratory. $TNF\alpha^{-/-}$ mice were bred to the wild-type mice to generate $TNF\alpha^{+/-}$ mice. $TNF\alpha^{+/-}$ mice were intercrossed to generate $TNF\alpha^{-/-}$ and $TNF\alpha^{+/+}$ mice, which were genotyped according to the method provided by the supplier. For diet studies, mice were fed either rodent chow (6% fat by weight and 12% of calories from fat, RD8664 Harlan Teklad, Madison, WI) or high fat diet (41% of calories from fat, 34% of calories from sucrose, TD88137 Harlan Teklad) for the indicated length of time. Body adiposity was determined by dual-energy x-ray absorptometry (DEXA, Lunar PIXImus mouse densitometer). All studies were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center and were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Treatment of Mice with Glucocorticoids or TNFα—For glucocorticoid treatment to induce insulin resistance, male 14-week-old FVB mice were injected intraperitoneally once per day with saline or 3, 30, or 300 μg of dexamethasone for 4 days and sacrificed 5 h after the last injection in the random-fed state. Blood glucose levels were measured using a One Touch II glucometer (Lifescan Inc., Johnson & Johnson, Milpitas, CA). Serum insulin levels were determined with a rat insulin enzyme-linked immunosorbent assay (ELISA) using mouse insulin standards (Crystal Chem Inc., Chicago, IL). For TNFα injection experiments, random-fed female FVB mice at 8–9 weeks of age were used. For PTP1B RNA measurements, mice were injected intravenously with saline or 3.3 μg of murine TNFα (Sigma, St. Louis, MO), and tissues were harvested from mice 4 h after injection. For PTP1B protein measurements, mice were injected intravenously with saline or 3.3 μg of murine TNFα, followed by a second intravenous injection of saline or TNFα 5 h later. Tissues were harvested from mice 9 h after the initial TNFα injection.

Cell Culture and 3T3-L1 Adipocyte Differentiation—All tissue culture reagents were from Invitrogen (Carlsbad, CA) unless otherwise indicated. HeLa cells were maintained in Dulbecco's modified Eagle medium with 5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C, 5% CO_2. H-4-II-E cells were maintained in Alpha's modification of Eagle media (Cellgro, Mediatech, Herndon, VA) with 5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C, 5% CO_2. 3T3-L1 fibroblasts (obtained from the ATCC, #CCL 92.1, Rockville, MD) were grown in Dulbecco's modified

Eagle medium with 10% newborn calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C, 5% CO_2. Two days after reaching confluence, fibroblasts were induced to differentiate by treatment with 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, and 1 μg/ml insulin (all from Sigma) for 3 days. During and following differentiation, Dulbecco's modified Eagle medium was supplemented with 10% fetal calf serum. Cells were used for experiments 14–21 days after induction of differentiation and only if >90% of cells showed fat droplets. HeLa cells, H-4-II-E cells, and 3T3-L1 adipocytes were treated with murine TNFα (Sigma) for the doses and times indicated in the figure legends. For inhibitor studies, cells were incubated with actinomycin D (2 μg/ml, Sigma), sodium salicylate (5 mM, Sigma), PD98059 (10 μM, Calbiochem), SB202190 (10 μM, Sigma), SP600125 (5 μM, Sigma), or without inhibitor for 1–1.5 h, followed by incubation with 20 ng/ml (1.2 nM) mouse TNFα (Sigma) or without cytokine for 4 h.

RNA Extraction and Gene Expression Analysis by Real-time Reverse Transcription-PCR—Total RNA was extracted from homogenized tissues and cultured cells using TriReagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. Quantitative real-time reverse transcription-PCR was performed for each sample in triplicate with 2.5 ng of total RNA, 1× TaqMan Universal Master Mix no AmpErase UNG, 6.25 units of murine leukemia virus reverse transcriptase (both from Applied Biosystems, Foster City, CA) and gene-specific primers-probe sets, using an MX4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). Reverse transcription reactions were carried out at 48 °C for 30 min, reactions were then denatured at 95 °C for 10 min, and cDNAs were amplified by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers and probes for 18S (which detects human and mouse 18S RNA) and suppressor of cytokine signaling 3 have been previously described (44, 45). Primer-probe sets for human PTP1B, murine PTP1B, TNFα, macrophage antigen CD68, and hypoxanthine guanine phosphoribosyltransferase (HPRT) were purchased as predesigned TaqMan gene expression assays and run as per the manufacturer's instructions (Applied Biosystems). Gene expression was determined by the standard curve method and normalized to 18S RNA as indicated. When the 18S RNA amount differed between experimental groups, gene expression was normalized to HPRT RNA. Accuracy of RNA quantitation was optimized by gene-specific primer-probe sets that span intron-exon boundaries and lack of amplification in no-RT and no-template controls.

Immunoblotting—Liver, perigonadal white adipose tissue, and hind limb skeletal muscle were dissected from mice and rats and frozen immediately in liquid nitrogen. Lateral hypothalamus, medial hypothalamus (containing the ventromedial hypothalamus and dorsomedial hypothalamus), and arcuate nucleus of hypothalamus were punched out of a 2 mM coronal or sagittal brain slice encompassing the hypothalamic region and frozen in liquid nitrogen. Tissues or cultured cells were homogenized in 20 mM Tris pH 7.4, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 1 min with a Polytron homogenizer, incubated at 4 °C for 40–60 min with rotation, and centrifuged for 10 min at 16,000 × *g*. Supernatant protein (50 μg) was separated by electrophoresis on 10% SDS-PAGE gels and immunoblotted (46) with monoclonal anti-human PTP1B (for human samples, EMD Biosciences, San Diego, CA), polyclonal anti-murine PTP1B (for mouse and rat samples) (8), polyclonal anti-extracellular-related kinases 1 and 2 (ERK1/2, gift of J. Blenis, Harvard Medical School, Boston, MA), or polyclonal anti-goat actin (Sigma) antibodies. To detect CD68 protein in adipose tissue lysates, 400 μg of protein was incubated with wheat germ agarose overnight and washed three times with lysis buffer, and then bound proteins were separated by SDS-PAGE on 7.5% gels and subjected to immunoblotting with polyclonal anti-mouse CD68 antibodies (Serotec, Raleigh, NC). Immunoblotted protein bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry using ImageQuaNT software (Molecular Dynamics/Amersham Biosciences) or direct chemiluminescence detection with GeneGnome (SynGene, Frederick, MD).

Chromatin Immunoprecipitation Assays—Immunoprecipitation of NF-κB bound to *PTP1B* gene chromatin was accomplished using a two-step cross-linking method (47). ChIP assays were performed on day 14–21 post-differentiation 3T3-L1 adipocytes treated with 20 ng/ml (1.2 nM) mouse TNFα (Sigma) or without cytokine for 4 h. ChIP assays were additionally performed on frozen livers of randomly fed 8–9-week-old female FVB mice harvested 4 h after intravenous injection of mice with saline or 3.3 μg of murine TNFα, as described above. After removal of media, adipocytes were washed three times with PBS prior to cross-linking. In addition, for adipocytes, all washing and cross-linking steps were carried out with the addition of 1 mM $MgCl_2$ to aid cell retention on plates. Cells or finely minced livers were fixed in disuccinimidyl glutarate added to a final concentration of 2 mM in PBS for 45 min at room temperature, washed three times with PBS, and then followed by fixation with 1% formaldehyde in PBS for 15 min at room temperature. Samples were washed three times in PBS and resuspended in 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS at room temperature and transferred to ice. Chromatin was sheared by sonication until average DNA length determined by gel electrophoresis was ~500–3000 bp. Samples were centrifuged (5000 × *g* for 5 min), and soluble chromatin was transferred to a new tube. Lysates, diluted in 1× RIPA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture (Sigma)), were precleared by incubation with 40 μl of protein G-agarose beads for 1 h at room temperature, immunoprecipitated with 4 μg of anti-p65 antibody (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA) or a nonspecific control antibody (CD68 antibody, Santa Cruz Biotechnology) overnight at 4 °C, and captured on protein G-agarose beads for 2 h at 4 °C, all with rotation. Immunocomplexes were washed three times in 1× RIPA buffer. Immunocomplexes and input (10% of samples used for immunoprecipitations) were resuspended in 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, and 0.5% SDS, incubated for 4 h at 65 °C, and extracted once with phenol-chloroform, and DNA in the aqueous phase was subjected to ethanol precipitation. Chromatin resuspended in H_2O was amplified by PCR using primers 5′-ACATGTCCCATCCGTTCATT-3′ and 5′-CATCTCCCACGTCTTGGAAT-3′, flanking

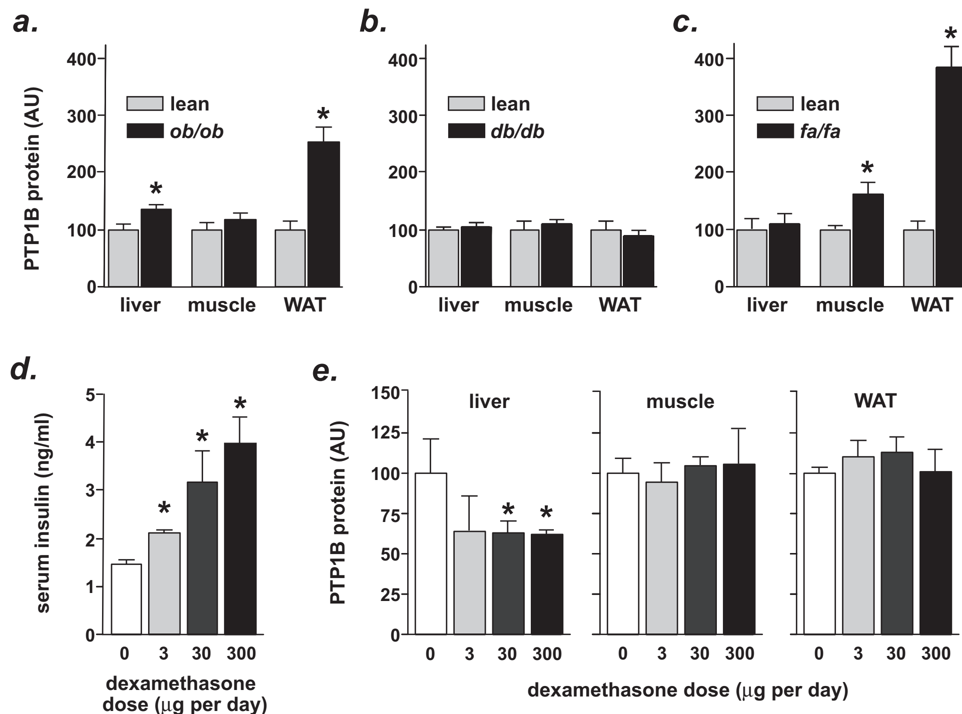

FIGURE 1. **PTP1B protein overexpression in insulin-target tissues is not regulated by obesity, insulin resistance, or diabetes *per se*.** *a–c*, PTP1B protein levels in liver, skeletal muscle, and perigonadal white adipose tissue (*WAT*) of 8- to 10-week old *ob/ob* mice (*a*), diabetic *db/db* mice (*b*), and Zucker *fa/fa* rats (*c*) and their respective lean controls were determined by immunoblotting. Results are means ± S.E. ($n = 4–7$ per group). *, $p \leq 0.05$ compared with WT animals by *t* test. *d*, serum insulin levels in 11-week-old female FVB mice treated with the indicated concentration of dexamethasone daily for 4 days. Results are means ± S.E. ($n = 3$ per group). *, $p \leq 0.05$ compared with untreated mice by one-way ANOVA. *e*, PTP1B protein levels in liver, skeletal muscle, and perigonadal WAT of dexamethasone-treated mice were determined by immunoblotting. Results are means ± S.E. ($n = 3$ per group). *, $p \leq 0.05$ compared with control or untreated mice by one-way ANOVA. For all experiments, PTP1B protein amount was normalized to the amount of ERK1/2 or other control proteins. Proteins were quantified by densitometry of x-ray films.

an NFκB site −892 to −883 relative to the start site of transcription of the *PTP1B* promoter region, predicted using MULAN software from the NCBI DCODE.org Comparative Genomics Center. Primers specific for sequences in the first intron of *PTP1B* were used as a negative control for NFκB binding. Duplicate DNA samples were amplified by PCR at an annealing temperature of 56 °C for 30 cycles. PCR products were separated by electrophoresis on agarose gels, visualized by ethidium bromide staining, and quantitated using Quantity One software (Bio-Rad Laboratories, Hercules, CA). All experiments were repeated at least twice.

Statistical Analyses—Data are expressed as means ± S.E. Statistical analyses were performed using StatView software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between groups were made by unpaired two-tailed Student *t* tests, or one-way or two-way ANOVA with post-hoc analyses, when appropriate, as indicated.

RESULTS

PTP1B Expression Is Regulated in Different Metabolic States and Not by Insulin Resistance, Obesity, or Diabetes per se—PTP1B overexpression has been reported in skeletal muscle, adipose tissue, and/or liver of several obese, insulin-resistant, or diabetic animals or patients (18–28) but not in others (24, 29–31). To determine whether these conflicting reports may be due to experimental differences and whether tissue PTP1B overexpression is characteristic of obese and diabetic states, we examined PTP1B tissue expression levels in three genetically obese, insulin-resistant, and/or diabetic animal models at young adult age (8–10 weeks old), as well as in chemically-induced insulin-deficient and insulin-resistant mice (Fig. 1). PTP1B was overexpressed 1.3-fold in liver and 3-fold in adipose tissue of obese, insulin-resistant *ob/ob* mice (Fig. 1*a*). PTP1B expression was normal in skeletal muscle of *ob/ob* mice (Fig. 1*a*). In contrast, PTP1B protein was not overexpressed in liver, skeletal muscle, or adipose tissue of obese, insulin-resistant, type 2 diabetic *db/db* mice compared with lean controls (Fig. 1*b*) (fasting blood glucose 51 ± 4 mg/dl for lean *versus* 360 ± 54 mg/dl for *db/db*, $p < 0.01$). PTP1B expression was also normal in liver of obese, insulin-resistant Zucker rats but overexpressed 1.5-fold in skeletal muscle and 3.7-fold in adipose tissue (Fig. 1*c*). Results are shown for 16-h fasted animals, but similar differences were observed in fed *ob/ob* mice tissues and Zucker rat adipose tissue, and were reported previously in studies of muscle from Zucker rats (18). We confirmed that PTP1B was overexpressed 1.5- to 2-fold in liver of lean streptozotocin-treated type 1 diabetic mice compared with untreated mice (blood glucose 131 ± 10 mg/dl for untreated *versus* 381 ± 27 mg/dl for streptozotocin, $p < 0.05$) and, as reported in rats (19), insulin treatment to normalize blood glucose did not reduce PTP1B levels (data not shown). To determine whether PTP1B overexpression accompanies insulin resistance in non-obese mice, we treated FVB mice with 3, 30, or 300 μg of dexamethasone daily for 4 days to induce insulin resistance (48). Blood glucose levels, body weight, and food intake did not change with dexamethasone treatment (data not shown). Serum insulin levels were increased in mice receiving all doses of dexamethasone treatment compared with untreated mice, indicating the development of insulin resistance (Fig. 1*d*). Surprisingly, dexamethasone treatment decreased PTP1B protein levels by ~35% in liver (Fig. 1*d*) compared with untreated mice. PTP1B levels were not changed in skeletal muscle and perigonadal adipose tissue by dexamethasone treatment. Together with previous studies (19–21, 27), our data demonstrate that PTP1B expression is dynamically regulated in different metabolic states. Furthermore, they suggest that PTP1B overexpression in liver, adipose tissue, and skeletal muscle is not determined simply by obesity, insulin resistance, or diabetes *per se*. Our observation that the potent anti-inflammatory glucocorticoid dexamethasone decreased

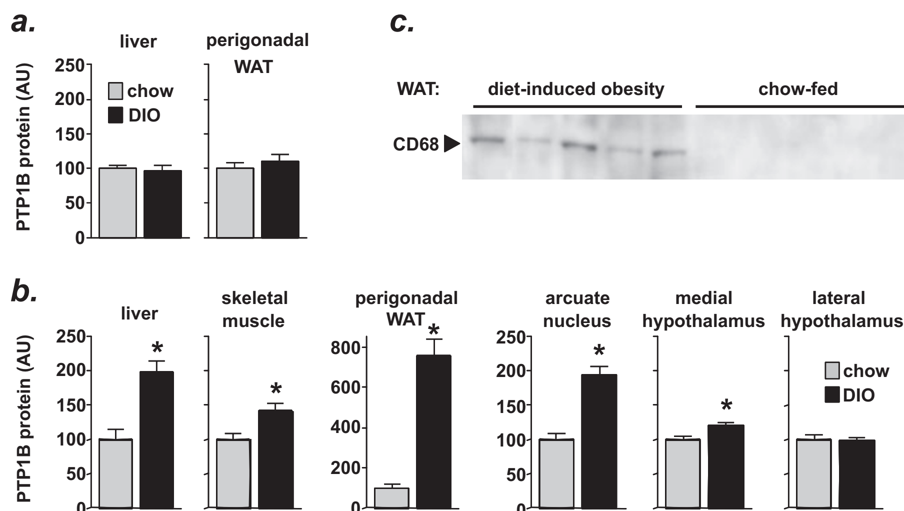

FIGURE 2. **Overexpression of PTP1B protein in insulin-target tissues and the arcuate nucleus of hypothalamus of mice with DIO coincides with inflammation in adipose tissue.** *a*, PTP1B protein levels in liver and perigonadal WAT of female FVB mice fed a low fat chow diet or a high fat diet from weaning until 15 weeks of age were determined by immunoblotting. PTP1B levels were normalized to ERK1/2 levels. Proteins were quantified by densitometry of x-ray films. Results are means ± S.E. ($n = 8$–9 per group). *b*, PTP1B protein levels in liver, skeletal muscle, perigonadal white adipose tissue (*WAT*), arcuate nucleus of hypothalamus, medial or lateral hypothalamus, of female FVB mice fed a low fat chow diet or a high fat diet from weaning until 20 weeks of age were determined by immunoblotting. Lateral hypothalamus, medial hypothalamus, and arcuate nucleus of the hypothalamus were dissected from a coronal brain slice. PTP1B levels were normalized to ERK1/2 levels for all tissues except WAT. For WAT, ERK1/2 levels were different between groups, and PTP1B levels were normalized to actin. PTP1B, ERK1/2, and actin were quantified by densitometry of x-ray films. Results are means ± S.E. ($n = 7$–8 per group). *, $p \leq 0.05$ compared with chow-fed animals by *t* test. *c*, CD68 protein levels in adipose tissue of female FVB mice fed a low fat chow diet or a high fat diet from weaning until 20 weeks of age. Proteins in perigonadal adipose tissue (*WAT*) lysates were detected by immunoblotting with polyclonal antibodies specific for mouse CD68. Each lane represents one animal.

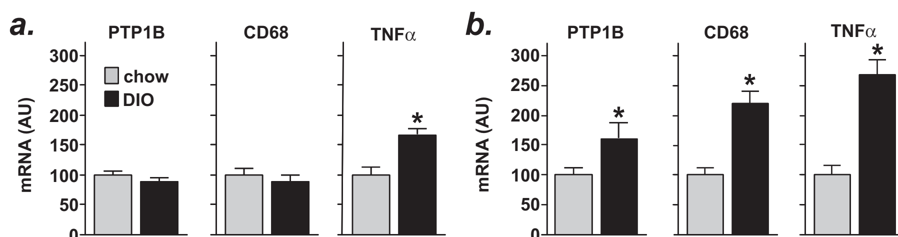

FIGURE 3. **Increased TNFα expression in adipose tissue accompanies PTP1B overexpression in insulin- and leptin-target tissues of mice with DIO.** PTP1B, TNFα, and CD68 mRNA levels from perigonadal adipose tissue of female FVB mice fed a low fat chow diet or a high fat diet from weaning until 15 weeks (*a*) or 20 weeks (*b*) of age. PTP1B, TNFα, and CD68 mRNA amounts were normalized to HPRT mRNA. PTP1B, TNFα, CD68, and HPRT mRNAs were measured by quantitative real-time reverse transcription-PCR. Results are means ± S.E. ($n =$ 7–9 per group). *, $p \leq 0.05$ compared with chow-fed animals by *t* test.

liver PTP1B despite causing insulin resistance in mice and recent work identifying inflammation as a common element in development of obesity and diabetes (33, 34, 49) led us to consider whether inflammation may help promote tissue PTP1B overexpression *in vivo*.

PTP1B Overexpression in Mice with Diet-induced Obesity Coincides with the Development of Inflammation—To determine whether PTP1B is overexpressed in insulin- and leptin-target tissues of mice with high fat diet-induced obesity (DIO) and when overexpression occurs, we fed FVB mice either rodent chow or a high fat diet from weaning and examined tissue PTP1B expression at different time points. At 15 weeks of age, body weight was increased by 18% ($p < 0.01$) in mice with DIO (28.17 ± 1.23 g) compared with chow-fed controls (23.97 ± 0.74 g), but PTP1B expression was unaffected in liver or adipose tissue of mice with DIO compared with chow-fed mice (Fig. 2*a*). At 20 weeks of age, body weight was increased 24% ($p < 0.001$) in mice with DIO (33.82 ± 1.28 g) compared with chow-fed controls (27.26 ± 0.66 g). Adiposity determined by DEXA analysis was also increased 60% ($p < 0.001$) in mice with DIO (34.8 ± 2.1%) compared with chow-fed controls (21.7 ± 1.5%). At this time, PTP1B was overexpressed by 2-fold in liver, 1.5-fold in skeletal muscle, and 7-fold in adipose tissue in mice with DIO compared with controls (Fig. 2*b*). PTP1B also was expressed at 2-fold higher than normal levels in arcuate nucleus of hypothalamus and to a lesser extent in ventromedial/dorsomedial hypothalamus but not in lateral hypothalamus (Fig. 2*b*). PTP1B overexpression coincided with increased expression of CD68, a macrophage marker (Fig. 2*c*), suggesting the development of diet-induced adipose inflammation (36, 37).

To determine the mechanisms mediating increased PTP1B protein expression in DIO, we measured PTP1B mRNA levels in adipose tissue of chow-fed mice and mice with DIO at 15 and 20 weeks of age. Adipose PTP1B mRNA levels were similar in chow-fed mice and mice with DIO at 15 weeks (Fig. 3*a*) but were increased in mice with DIO compared with chow-fed controls at 20 weeks (Fig. 3*b*). These results suggest that PTP1B expression is regulated at the level of transcription and/or mRNA stability in adipose tissue of mice with DIO, consistent with previous findings in diabetic animal models (19). CD68 mRNA levels were increased in adipose tissue of mice with DIO compared with chow-fed mice at 20 weeks of age (Fig. 3*b*), but not at 15 weeks of age (Fig. 3*a*), consistent with the increased expression of CD68 protein observed in adipose tissue of mice with DIO at 20 weeks of age (Fig. 2*c*). TNFα mRNA levels were increased in adipose tissue of mice with DIO compared with chow-fed mice at both 15 and 20 weeks, but the magnitude of increase was substantially higher in mice at 20 weeks (Fig. 3, *a versus b*). These data led us to hypothesize that tissue PTP1B overexpression in mice with DIO may be mediated by pro-inflammatory factors associated with adipose tissue inflammation.

TNFα Positively Regulates Expression of PTP1B in Cultured Cells and Insulin- and Leptin-target Tissues of Mice—To determine whether the increased TNFα expression observed in adi-

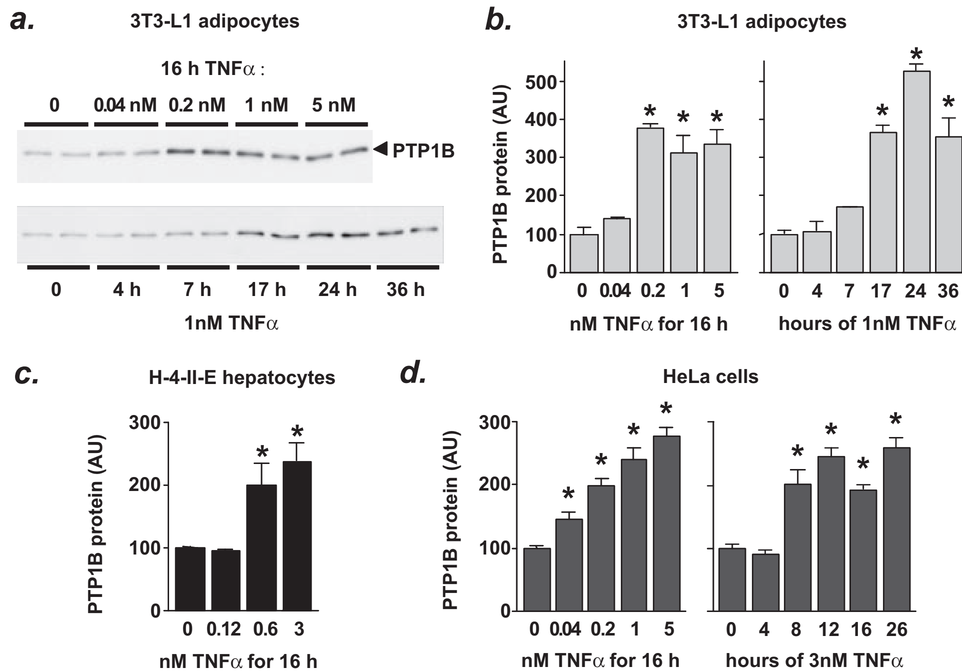

FIGURE 4. **TNFα treatment increases PTP1B protein expression in multiple cultured cell types.** PTP1B protein amount in lysates of three different cell types treated with TNFα was measured by immunoblotting. *a* and *b*, 14–21-day post-differentiation 3T3-L1 adipocytes were incubated with the indicated concentrations of TNFα for 16 h (*a*, *top panel* and *b*, *left panel*) or with 1 nM TNFα for the indicated times (*a*, *bottom panel* and *b*, *right panel*). Representative immunoblots for PTP1B (*a*) and means ± S.E. ($n = 2$–3 per condition) (*b*) are shown. *c*, PTP1B protein from H-4-II-E hepatoma cells incubated with the indicated concentrations of TNFα for 16 h. Results are means ± S.E. ($n = 3$ per condition). *d*, PTP1B protein from HeLa cells incubated with the indicated concentrations of TNFα for 15–16 h (*left*) or with 3 nM TNFα for the indicated times (*right*). Results are means ± S.E. ($n = 3$ per condition). For all experiments, PTP1B and ERK1/2 proteins were quantified by densitometry of x-ray films or direct chemiluminescence detection and PTP1B was normalized to ERK1/2. *, $p \leq 0.05$ compared with cells without TNFα by one-way ANOVA.

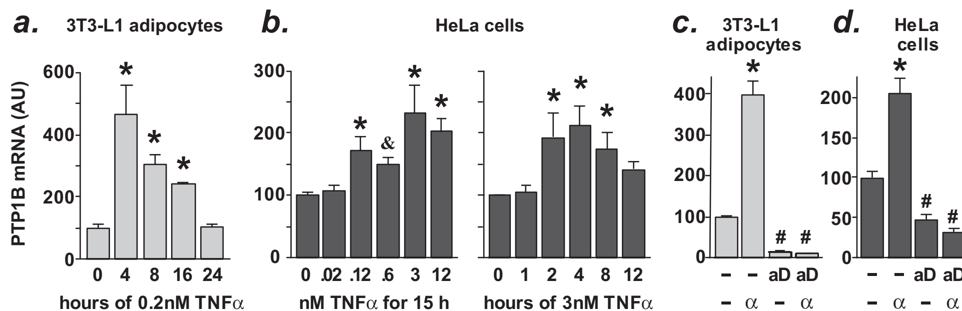

FIGURE 5. **TNFα treatment increases PTP1B mRNA expression in cultured cells via transcriptional transactivation.** *a*, PTP1B mRNA from 14–21-day post-differentiation 3T3-L1 adipocytes incubated with 0.2 nM TNFα for the indicated times is shown. Results are means ± S.E. ($n = 2$ per condition). *b*, PTP1B mRNA from HeLa cells incubated with the indicated concentrations of TNFα for 15 h (*left*) or with 3 nM TNFα for the indicated times (*right*) is shown. Results are means ± S.E. ($n = 3$ per condition). *, $p \leq 0.05$ and &, $p \leq 0.1$ compared with untreated cells by one-way ANOVA. *c* and *d*, PTP1B mRNA from 14–21-day post-differentiation 3T3-L1 adipocytes (*c*) or HeLa cells (*d*) treated with actinomycin D (*aD*) (2 μg/ml) or without inhibitor for 1 h before incubation with 1.2 nM TNFα or without cytokine for 4 h. Results are means ± S.E. ($n = 6$ in *c* and $n = 12$ in *d*, per condition). *, $p \leq 0.05$ compared with the corresponding condition without TNFα; and #, $p \leq 0.05$ compared with the corresponding condition without actinomycin D by two-way ANOVA . For all experiments, PTP1B mRNA and control 18S rRNA were measured by real-time quantitative reverse transcription-PCR and PTP1B mRNA was normalized to 18S rRNA.

pose tissue of mice with DIO could mediate PTP1B overexpression, we treated differentiated 3T3-L1 adipocytes and H-4-II-E hepatoma cells, as well as HeLa cells, with murine TNFα and measured expression of PTP1B protein. TNFα treatment increased PTP1B protein expression 3- to 5-fold in 3T3-L1 adipocytes in a dose- and time-dependent manner (Fig. 4, *a* and *b*). Similarly, TNFα treatment increased PTP1B protein levels by 2-fold in H-4-II-E hepatoma cells (Fig. 4*c*). TNFα also increased PTP1B protein levels by 2- to 3-fold in HeLa cells, in a dose- and time-dependent manner (Fig. 4*d*). Similar results were observed in serum-starved and unstarved HeLa cells and in HeLa cells treated with murine and human TNFα (not shown). Increased expression of PTP1B was observed in all three cell types between 8 and 36 h post-treatment at TNFα doses known to regulate expression of other TNFα-responsive genes (50). These data identify TNFα as a positive regulator of PTP1B expression in diverse cell types, including insulin-responsive 3T3-L1 adipocytes and H-4-II-E hepatoma cells.

To assess the mechanisms mediating TNFα induction of PTP1B protein expression, we measured PTP1B mRNA levels in cultured cells treated with TNFα. In 3T3-L1 adipocytes, TNFα treatment increased PTP1B mRNA up to 5-fold with a peak expression at 4 h (Fig. 5*a*). This effect was also dose-dependent (data not shown). Similarly, TNFα treatment increased PTP1B mRNA expression 2- to 2.5-fold in HeLa cells (Fig. 5*b*). Pretreatment of 3T3-L1 adipocytes and HeLa cells with the RNA polymerase II inhibitor actinomycin D reduced PTP1B mRNA levels in both 3T3-L1 adipocytes and HeLa cells as expected and prevented TNFα-mediated PTP1B mRNA expression (Fig. 5, *c* and *d*). The delay in PTP1B protein overexpression compared with PTP1B mRNA upon TNFα treatment likely reflects the long half-life of PTP1B protein (≥6–8 h) (51, 52) compared with mRNA (∼3 h in HeLa cells and 1.5 h in 3T3-L1 adipocytes, Fig. 5, *c* and *d*). These data suggest that TNFα regulates PTP1B expression at the transcriptional level.

TNFα increases the expression of inflammatory genes via several signaling pathways, including the NFκB pathway and/or one or more mitogen-activated kinases, including c-Jun N-terminal kinase (JNK), p38, and ERK1/2 (43). To determine which of these pathways mediates increased PTP1B expression, we pretreated 3T3-L1 adipocytes with high dose salicylate to prevent NFκB activation, PD98059 to prevent ERK activation,

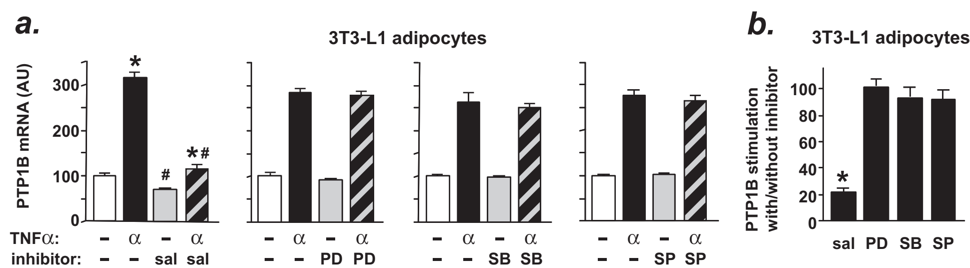

FIGURE 6. **TNFα regulates PTP1B expression in part via transcription in 3T3-L1 adipocytes.** *a*, PTP1B mRNA from 14-day post-differentiation 3T3-L1 adipocytes incubated with sodium salicylate (*sal*, 5 mM), PD98059 (*PD*, 10 μM), SB202190 (*SB*, 10 μM), SP600125 (*SP*, 5 μM), or without inhibitor for 1 h, followed by incubation with 1.2 nM TNFα or without cytokine for 4 h is shown. Results are means ± S.E. ($n = 6$ per condition). *, $p \leq 0.05$ compared with the corresponding condition without TNFα and #, $p \leq 0.05$ compared with the corresponding condition without inhibitor by one-way ANOVA. *b*, data from *a* represented as the increment of increase in PTP1B expression with TNFα treatment in the presence of inhibitor divided by the increment in the absence of inhibitor. PTP1B mRNA and control 18S rRNA were measured by real-time quantitative PCR, and PTP1B mRNA was normalized to 18S rRNA.

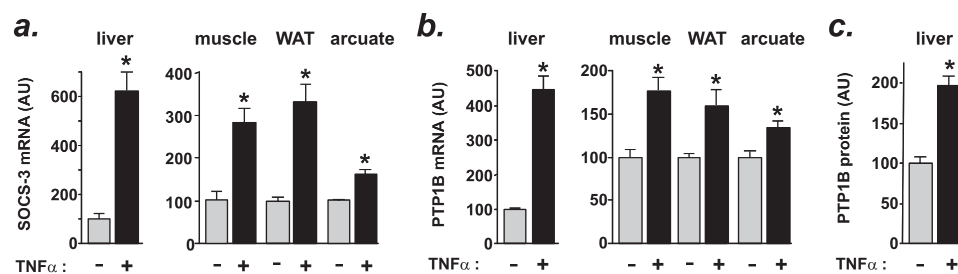

FIGURE 7. **Acute TNFα treatment increases PTP1B expression in insulin- and leptin-target tissues of mice.** *a* and *b*, FVB mice were injected intravenously with saline or TNFα (3.3 μg per mouse) and sacrificed 4 h later. PTP1B mRNA, suppressor of cytokine signaling 3 (*SOCS-3*) mRNA, and 18S rRNA from liver, gastrocnemius skeletal muscle, perigonadal adipose tissue (*WAT*), and arcuate nucleus of hypothalamus were measured by real-time quantitative reverse transcription-PCR. SOCS-3 (*a*) or PTP1B (*b*) mRNA was normalized to 18S rRNA. Results are means ± S.E. ($n = 6$ per condition). *c*, FVB mice were injected intravenously with saline or 3.3 μg of TNFα, followed by a second intravenous injection of the same 5 h later. Tissue was harvested from mice 9.5 h after the initial TNFα injection. PTP1B and ERK1/2 protein amount in liver was determined by immunoblotting. PTP1B and ERK1/2 proteins were quantified by densitometry of x-ray films or direct chemiluminescence detection and PTP1B amount was normalized to the amount control proteins ERK1/2. Results are means ± S.E. ($n = 6$ per condition). *, $p \leq 0.05$ compared with untreated mice by *t* test.

SB202190 to prevent p38 activation, or SP600125 to prevent JNK activation, prior to treatment with TNFα. Treatment with salicylate alone reduced PTP1B mRNA expression by ~30% (Fig. 6*a*, *panel 1*). Salicylate pretreatment of adipocytes also reduced TNFα-induced PTP1B mRNA expression by ~65% compared with adipocytes treated with TNFα alone (Fig. 6*a*, *panel 1*). Basal and TNFα-induced PTP1B expression were similar in adipocytes pretreated with PD98059, SB202190, or SP600125 and cells without inhibitor pretreatment (Fig. 6*a*, *panels 2–4*). Thus, salicylate reduced the increment of TNFα-induced PTP1B mRNA expression above basal levels in 3T3-L1 adipocytes by ~80% (Fig. 7*b*). In HeLa cells, treatment with SB202190 reduced the increment of TNFα-induced PTP1B mRNA expression above basal levels by ~40% compared with cells without inhibitor pretreatment (not shown), whereas treatment with salicylate did not affect the increment of TNFα-induced PTP1B mRNA expression above basal levels. Together, these results suggest that TNFα may regulate PTP1B expression via multiple signaling pathways in different cell types.

To ask whether TNFα can promote PTP1B overexpression in insulin- and leptin-target tissues of mice, we treated FVB mice with TNFα. Because TNFα is known to be rapidly cleared after injection in mice (53, 54), we injected a high dose of TNFα to sustain high serum levels. Four hours after treatment, PTP1B mRNA levels were increased by 4.5-fold in liver, 1.8-fold in skeletal muscle, 1.6-fold in adipose tissue, and 1.4-fold in the arcuate nucleus. These increases (Fig. 7*b*) are similar to those seen in the expression of suppressor of cytokine signaling 3 (SOCS-3) (Fig. 7*a*), a gene known be regulated by TNFα. PTP1B protein was increased 1.25-fold in liver of TNFα-treated mice compared with control mice (data not shown). Because increased PTP1B mRNA precedes increased PTP1B protein by several hours in cultured cells treated with TNFα (Figs. 4 and 5), and injected TNFα is cleared within minutes to hours in mice (53, 54), we injected mice twice with TNFα at 5-h intervals. PTP1B protein was increased 2-fold in liver of these mice (Fig. 7*c*), demonstrating that TNFα is sufficient to increase PTP1B protein amount in an insulin- and leptin-target tissue.

To determine whether TNFα-induced NFκB could transactivate *PTP1B* expression in cultured cells and insulin- and leptin-target tissues in mice, we examined binding of NFκB subunit p65 to the mouse *PTP1B* promoter in cultured cells and tissues of mice treated with or without TNFα. NFκB subunit p65 transactivates transcription of inflammatory genes in response to TNFα (55). We analyzed the mouse *PTP1B* promoter for potential NFκB binding sites using MULAN software from NCBI DCODE.org Comparative Genomics Center. This analysis identified a potential NFκB binding site, 5′-TGGACTTTCC-3′, located −892 to −883 relative to the start site of transcription (Fig. 8*a*). After precipitation of cross-linked DNA with antibodies to p65 or a control antibody, precipitated DNA was amplified with primers flanking this potential NFκB binding site or a control sequence from the first intron of *PTP1B*. TNFα induced a 2-fold increase in recruitment of p65 to the *PTP1B* promoter in 3T3-L1 adipocytes compared with untreated cells (Fig. 8*b*). No differences between basal and TNFα conditions were observed in PCR product amount from control antibody immunoprecipitates or control PCR amplifications of downstream *PTP1B* intron sequences immunoprecipitated with either p65 or control antibodies. Similarly, TNFα induced a 2-fold increase in recruitment of p65 to the *PTP1B* promoter in liver of mice compared with untreated animals (Fig. 8, *c* and *d*). Again, no differences in PCR product amount between saline- and TNFα-treated mice were observed from control antibody immunoprecipitates or control PCR amplifications of downstream *PTP1B* intron

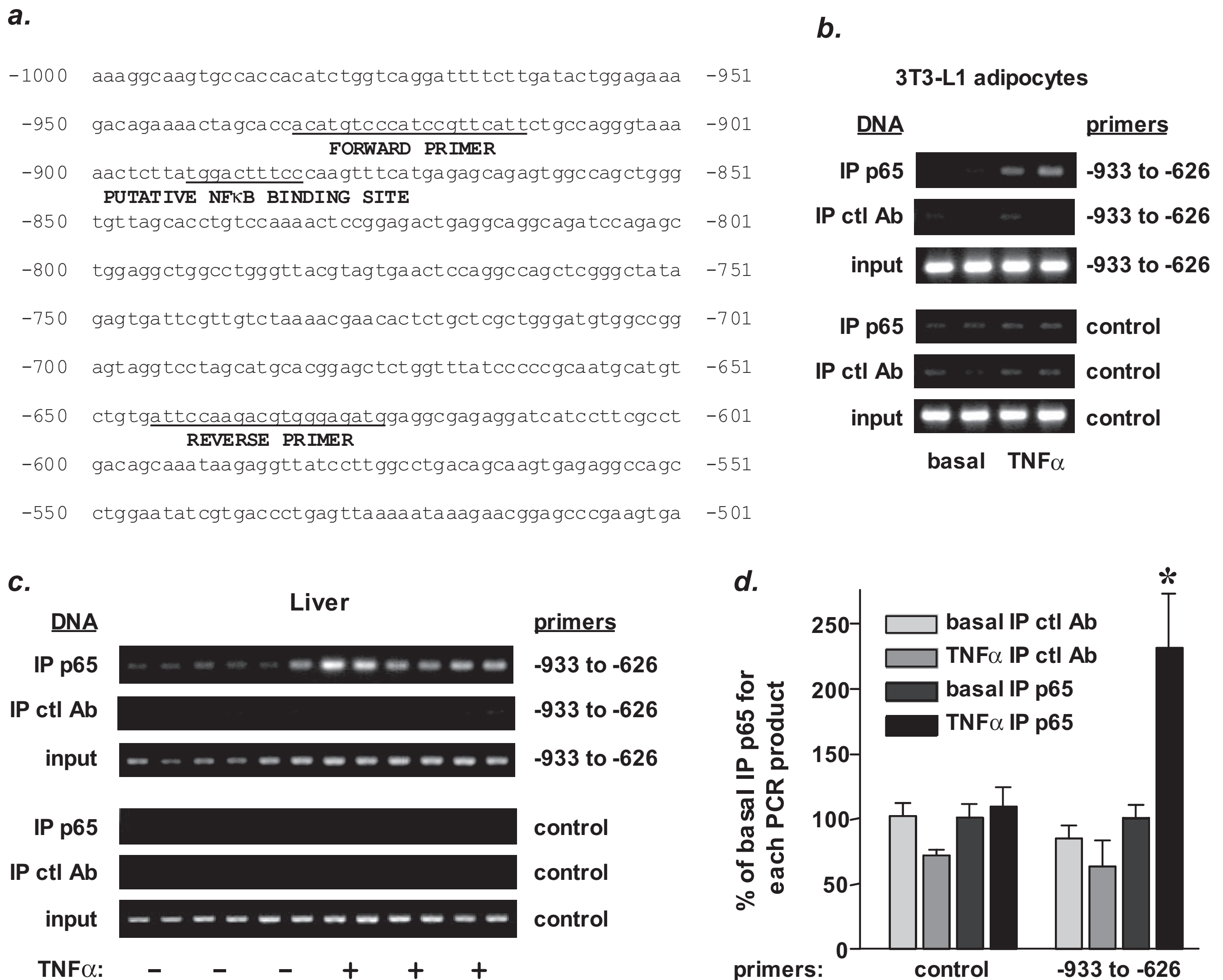

FIGURE 8. **TNFα induces NFκB subunit p65 binding to the mouse *PTP1B* promoter in 3T3-L1 adipocytes *in vitro* and mouse liver *in vivo*.** *a*, the sequence of the mouse *PTP1B* promoter from −1000 to −501 bp upstream of the start site of transcription is shown. The positions of the putative NFκB p65 binding site and primers used for ChIP analysis are indicated. ChIP was performed on chromatin from 3T3-L1 adipocytes (*b*) and liver of mice (*c*) treated with saline (basal) or TNFα for 4 h. Chromatin was immunoprecipitated (*IP*) with antibodies specific for the NFκB subunit p65 or control antibodies (ctl Ab). PCR products were amplified from input or immunoprecipitated samples with primers encompassing −933 to −626 of the *PTP1B* promoter or control primers specific for sequences in the first intron of *PTP1B*. Shown are duplicate PCR products, separated by agarose gel electrophoresis and visualized by ethidium bromide staining. *d*, quantitation of the results presented in *c*. Results are means ± S.E. of immunoprecipitated PCR products normalized to input PCR products for each sample ($n = 4$ per group). *, $p \leq 0.05$ compared with saline-treated mice.

sequences immunoprecipitated with either p65 or control antibodies. These data show that activation of NFκB p65 by TNFα in 3T3-L1 adipocytes *in vitro* and in mouse liver *in vivo* leads to the recruitment of p65 to the *PTP1B* promoter. Taken together with our data showing actinomycin D and salicylate inhibit PTP1B expression by TNFα, these data suggest that TNFα induces PTP1B expression in part by transcriptional transactivation via NFκB.

TNFα Deficiency Blunts Diet-induced PTP1B Overexpression in Adipose Tissue of Mice—To determine whether TNFα is necessary for PTP1B overexpression in insulin- and leptin-target tissues in mice with DIO, we compared the level of PTP1B overexpression in *TNFα*-deficient ($TNF\alpha^{-/-}$) and wild-type ($TNF\alpha^{+/+}$) mice with DIO. Lean and obese $TNF\alpha^{-/-}$ mice were previously reported to have increased insulin sensitivity compared with wild-type mice, due in part to enhanced insulin receptor signaling (56, 57). $TNF\alpha^{-/-}$ and $TNF\alpha^{+/+}$ mice on a mixed C57BL/6J × 129SvJ background were fed either normal rodent chow or a high fat diet from 6 weeks of age. For both male and female mice, body weights of mice with DIO were greater than chow-fed mice, but body weights of $TNF\alpha^{-/-}$ mice were not different from control $TNF\alpha^{+/+}$ mice on either diet (Fig. 9*a*). Random fed blood glucose levels were similar among all female and among all male mouse cohorts (Fig. 9*b*). For both male and female mice, random-fed serum insulin of $TNF\alpha^{-/-}$ mice was not statistically different from control $TNF\alpha^{+/+}$ mice on either diet, and DIO increased insulin levels in both genotypes (Fig. 9*c*). Wide variation in individual insulin values within groups was observed. TNFα was an important determinant of PTP1B expression level in adipose tissue of mice with DIO. In female mice, PTP1B expression in perigonadal, perirenal, and subcutaneous adipose tissue was similar in

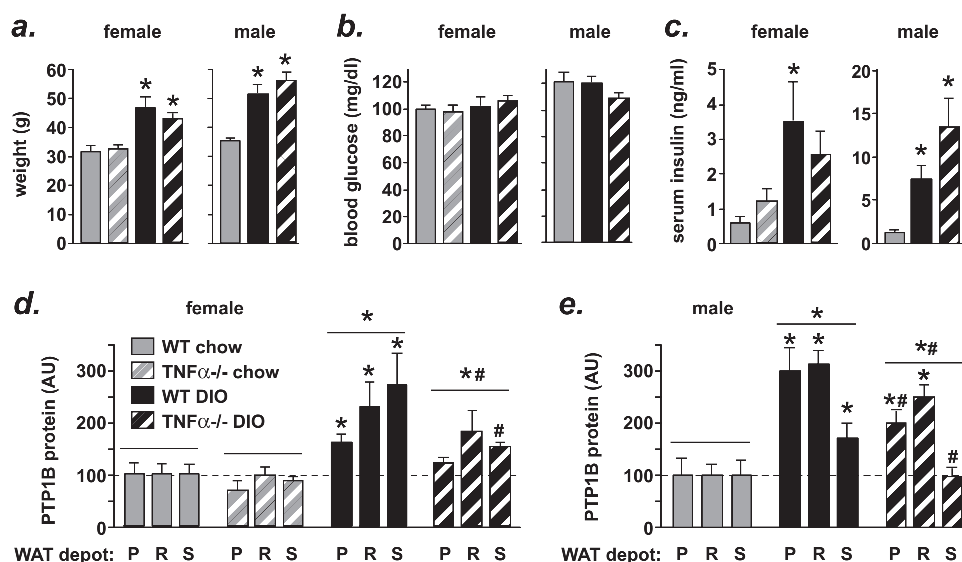

FIGURE 9. ***TNFα* deficiency blunts PTP1B overexpression in adipose tissue of mice with diet-induced obesity (DIO).** *TNF$\alpha^{+/+}$* (wild-type, *WT*) and *TNF$\alpha^{-/-}$* mice were fed either a low fat chow diet or a high fat diet from 6 weeks of age for 38 weeks for female mice or 26 weeks for male mice. Body weight (*a*), random-fed blood glucose (*b*), and serum insulin (*c*) were measured. Results are means ± S.E. (n = 6–8 per group). *, $p \leq 0.05$ compared with WT chow-fed mice by one-way ANOVA. *d* and *e*, PTP1B protein was measured in perigonadal (*P*), perirenal (*R*), and subcutaneous (*S*) adipose tissue of chow-fed mice and mice with DIO. PTP1B was detected by immunoblotting and quantified by densitometry. Results are means ± S.E. of PTP1B levels in perigonadal, subcutaneous, or perirenal adipose tissue depots (n = 5–8 per group for each adipose depot). Because diet or *TNFα* deficiency altered levels of several internal control proteins, PTP1B levels were measured from equivalent amounts of protein, which were verified by Ponceau S staining of immunoblots. *, $p \leq 0.05$ compared with WT chow-fed mice and #, $p \leq 0.05$ compared with WT DIO mice. *Symbols above bars* indicate differences determined by one-way ANOVA. *Symbols above lines* indicate differences determined by two-way ANOVA, with mouse group and adipose depot being the two factors.

chow-fed *TNF$\alpha^{+/+}$* and *TNF$\alpha^{-/-}$* mice (Fig. 9*d*). Adipose tissue PTP1B expression was increased by 1.6- to 2.7-fold in female *TNF$\alpha^{+/+}$* mice with DIO compared with *TNF$\alpha^{+/+}$* chow-fed mice. PTP1B overexpression in *TNF$\alpha^{-/-}$* mice with DIO was decreased by 44–71% compared with *TNF$\alpha^{+/+}$* mice with DIO. Similar results were observed in adipose tissue of male mice. Adipose tissue PTP1B expression was increased by 1.7- to 3.1-fold in male *TNF$\alpha^{+/+}$* mice with DIO compared with *TNF$\alpha^{+/+}$* chow-fed mice (Fig. 9*e*). PTP1B overexpression in male *TNF$\alpha^{-/-}$* mice with DIO was decreased 29–100% compared with *TNF$\alpha^{+/+}$* mice with DIO. Due to the variability of PTP1B expression in mice of this mixed genetic background, the decreases in PTP1B expression in *TNF$\alpha^{-/-}$* mice were not statistically significant for all individual adipose depots of female and male mice (Fig. 9, *d* and *e*) compared with *TNF$\alpha^{+/+}$* mice by one-way ANOVA. However, when PTP1B amount in all three adipose depots was compared among the different mouse cohorts by two-way ANOVA (with mouse group and adipose depot being the factors), PTP1B overexpression in *TNF$\alpha^{-/-}$* mice was significantly lower in both female ($p \leq$ 0.005) and male ($p \leq 0.002$) mice (Fig. 9, *d* and *e*) compared with *TNF$\alpha^{+/+}$* mice. Thus, TNFα deficiency in mice partly blunts diet-induced PTP1B overexpression in adipose tissue, suggesting that increased levels of TNFα in obese mice helps to promote increased PTP1B expression and thereby insulin resistance. Conversely, the lower levels of PTP1B overexpression in *TNF$\alpha^{-/-}$* mice with DIO may contribute to their enhanced insulin sensitivity (56, 57).

Unlike in FVB mice, PTP1B was not overexpressed in skeletal muscle of C57Bl/6J × 129SvJ mice with DIO. PTP1B overexpression in liver of C57Bl/6J × 129SvJ mice with DIO was higher than FVB mice with DIO, but varied up to 10-fold in both *TNF$\alpha^{+/+}$* and *TNF$\alpha^{-/-}$* mice. Both differences may be due to different strain backgrounds of the mice. Thus, we were unable to determine the effect of TNFα on diet-induced PTP1B overexpression in other tissues of these mice. Future studies on inbred genetic backgrounds may provide additional evidence of effects of TNFα on PTP1B overexpression in other tissues in obesity and other disease states.

DISCUSSION

Our results, together with previous studies (3), indicate that PTP1B expression is dynamically regulated by different metabolic states and that this regulation is an important mechanism by which PTP1B negatively regulates signaling pathways involved in growth and metabolism. Our results show that PTP1B is overexpressed in multiple insulin- and leptin-responsive tissues in mice with diet-induced obesity, including the arcuate nucleus and medial hypothalamus, important sites of PTP1B action on body weight regulation (11). These data and the effects of transgenic or adenovirus-mediated PTP1B expression (13, 14) suggest that diet-induced PTP1B overexpression contributes to the pathogenesis of insulin resistance in liver, adipose tissue, and muscle, while also exacerbating obesity through negative regulation of leptin action in hypothalamus. Our data suggest that PTP1B overexpression does not mediate the onset of diet-induced insulin resistance in mice but indicate it may be involved in the escalation of insulin resistance, which occurs when obesity-associated adipose tissue inflammation and increased TNFα expression arise (37). Importantly, PTP1B is also a key contributor to TNFα-induced insulin resistance, as recent work has shown PTP1B-deficiency ameliorates TNFα-induced insulin resistance in skeletal muscle (58). Because diet is a key contributing factor to most forms of human obesity and because adipose tissue inflammation accompanies obesity in humans (36), these data further support ongoing work to develop PTP1B inhibitors for the treatment and prevention of obesity and type 2 diabetes.

Nevertheless, reports of PTP1B expression levels in insulin-resistant, obese, and/or diabetic animals and humans have been inconsistent. Our data confirm that PTP1B is overexpressed in insulin-target tissues of several, but not all, insulin-resistant, obese, and diabetic animal models. In mice treated with glucocorticoids to induce insulin resistance, PTP1B expression

was unchanged in skeletal muscle and adipose tissue and decreased in liver. PTP1B is overexpressed in some insulin- and leptin-target tissues of young obese *ob/ob* mice and Zucker rats, but not *db/db* mice, despite their obesity. Glucose toxicity seems unlikely to explain the lack of PTP1B overexpression in *db/db* tissues, because PTP1B is overexpressed in several other type 1 and type 2 diabetic animal models (data not shown) (19–21). Together, these data indicate that PTP1B overexpression in insulin- and leptin-target tissues is not regulated simply by obesity, insulin resistance, or diabetes *per se* but suggest other factors regulate tissue PTP1B overexpression in these conditions.

The factors mediating PTP1B overexpression in certain obese and diabetic states have been unclear. Factors that cause or accompany development of insulin and leptin resistance are attractive potential mediators of PTP1B overexpression in obesity. Potential factors could include insulin, leptin, glucose, free fatty acids, glucocorticoids, and pro-inflammatory cytokines (33, 59, 60). Insulin, leptin, and glucose induce modest increases (1.35- to 2-fold) in PTP1B expression in cultured cells (27, 29, 61). It remains unclear whether these factors could mediate sustained PTP1B overexpression in insulin-resistant, leptin-resistant, and/or glucose-intolerant, obese, or diabetic states. Free fatty acids have additionally been reported to increase PTP1B expression in cells (62). In diet-induced obesity, however, initial elevations in free fatty acid levels precede PTP1B overexpression by several weeks (63), suggesting that additional factors might be necessary to mediate PTP1B overexpression *in vivo*.

Our work identifies TNFα as a positive regulator of PTP1B expression in diverse cell lines and tissues *in vivo*, and thus reveals that *PTP1B* is an inflammation-responsive gene. In agreement with this, we find that increased expression of macrophage markers and TNFα accompanies PTP1B overexpression in insulin- and leptin-responsive tissues during diet-induced adipose tissue inflammation and obesity *in vivo*. Conversely, despite inducing insulin resistance, glucocorticoids, which have anti-inflammatory effects, lower liver PTP1B levels and do not affect adipose and muscle PTP1B levels. TNFα was previously reported to have no (64, 65) or a transient (50) effect on PTP1B expression in cultured cells. Although all cells express TNFα receptors and NFκB subunits, and respond to TNFα *in vivo*, some immortalized and transformed cell lines may have altered TNFα responsiveness due to higher or constitutive activation of NFκB (66). In mice, TNFα treatment produced greater increases in PTP1B expression in liver compared with adipose tissue, skeletal muscle, and arcuate nucleus of hypothalamus. These results are consistent with previous studies showing that liver is a major target of systemically administered TNFα (67). In contrast, in mice with diet-induced obesity, PTP1B overexpression was greater in adipose tissue than liver, skeletal muscle, and arcuate nucleus. Because adipose tissue is the major source of increased TNFα in obesity (38), paracrine effects of TNFα on PTP1B expression may produce greater increases in PTP1B expression in adipose tissue in obesity than by systemic administration of TNFα. Our data cannot exclude that TNFα stimulates PTP1B expression by altering circulating levels of leptin, insulin, glucose, or other parameters *in vivo*. However, our data showing TNFα stimulates PTP1B overexpression in cultured 3T3-L1 adipocytes, H-4-II-E hepatocytes, and HeLa cells, which do not secrete insulin, glucose, or (except for 3T3-L1 adipocytes) leptin in response to TNFα, suggest that direct regulation of PTP1B expression by TNFα is possible in various cell types. Obesity-associated changes in expression and/or activity of tumor necrosis factor-α converting enzyme (TACE) and its inhibitor, tissue inhibitor of metalloproteinases-3 (TIMP-3) (68, 69), which are important regulators of TNFα processing and inflammation, may also contribute to TNFα's regulation of PTP1B expression in obesity.

Our data suggest that TNFα regulates PTP1B expression in part via NFκB activation in 3T3-L1 adipocytes and p38 activation in HeLa cells (not shown). Differences in the TNFα signaling pathways, which mediate PTP1B overexpression in HeLa cells and 3T3-L1 adipocytes, may reflect constitutive NFκB activation in HeLa cells, low p38 expression levels in 3T3-L1 adipocytes, or other differences between these cell lines (66, 70, 71). Although we cannot exclude that TNFα may stimulate PTP1B expression via increasing PTP1B mRNA stability, our data showing actinomycin D blocks and salicylate inhibits TNFα-induced PTP1B expression and TNFα recruits NFκB p65 to the mouse *PTP1B* promoter in 3T3-L1 adipocytes and mouse liver strongly suggest TNFα induces PTP1B expression at least in part via transactivation by NFκB. NFκB p65 plays an important role in gene transactivation during inflammation (55).

TNFα is sufficient to increase PTP1B expression in tissues *in vivo*, and increased expression of TNFα accompanies PTP1B overexpression in insulin- and leptin-responsive tissues in diet-induced obesity, but TNFα is likely not the only factor promoting PTP1B overexpression in obesity. Our data show that *TNFα* deficiency partially blunts diet-induced PTP1B overexpression in adipose tissue of mice, suggesting that tissue PTP1B overexpression in diet-induced obesity is partially mediated by TNFα. Activation of NFκB and p38 signaling by other pro-inflammatory cytokines that are elevated in obesity, such as interleukin-1β, or other factors may contribute to PTP1B overexpression in obesity in the absence of TNFα.

Adipose tissue inflammation is reported in genetic models of obesity at older ages than we examined (36, 37), suggesting that PTP1B expression may increase in older animals with adipose tissue inflammation as we observed in mice with diet-induced obesity. Additionally, leptin itself has inflammatory properties (72), so genetic absence of functional leptin or leptin receptor signaling in obese animals may alter development of adipose tissue inflammation itself and/or tissue PTP1B expression independent of or in addition to other inflammatory factors (29). In addition to obese, insulin-resistant animals and humans, PTP1B overexpression has been reported in liver and skeletal muscle of rats rendered insulin-deficient by streptozotocin treatment (19, 21). The pro-inflammatory cytokines TNFα and interleukin-1β and free radicals play an essential role in destruction of pancreatic β cells in type 1 diabetes (49). Lack of TNFα has been shown to prevent hyperglycemia and insulitis in several experimental diabetes models (49). Similarly, in streptozotocin-induced diabetes, increased TNFα secretion from peritoneal and splenic macrophages is observed (73, 74). Additionally, elevated levels of TNFα and other pro-inflamma-

tory cytokines have been proposed to be a causal link between obesity and type 2 diabetes (33, 34). Thus, increased expression of TNFα or other pro-inflammatory cytokines due to developing inflammation may be a unifying theme underlying the onset of PTP1B overexpression in diet-induced and genetic forms of obesity, and type 1 and 2 diabetes. Variation in inflammation onset, location, or relative degree or the inflammatory/anti-inflammatory milieu in different animal cohorts and patient populations due to diet, environment, treatments, or stress may explain the absence of PTP1B overexpression in insulin resistance, obesity, and diabetes in some studies. Our work identifying TNFα and inflammation as factors that regulate PTP1B expression *in vivo* demonstrates the types of insulin resistance (*i.e.* those caused by inflammation) in which PTP1B overexpression may be expected to play a causal role.

Although PTP1B is overexpressed in several tissues in obesity, PTP1B overexpression has also been reported in several tumors and tumor-derived cell lines (3). Inflammation and NFκB activation are a common elements of the pathology of both obesity (34, 75) and cancer (76) and may be a common element in the etiology of diabetes and malignant disease. Our data showing TNFα can increase tissue PTP1B expression and NFκB recruitment to the *PTP1B* promoter *in vivo*, together with observations that TNFα and NFκB activation are elevated in obesity and tumors, suggests a potential common mechanism for PTP1B overexpression in these pathologies. Further studies are needed to determine whether PTP1B overexpression is characteristic of other inflammatory diseases and could be ameliorated by anti-inflammatory therapies.

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