PROTEIN TYROSINE PHOSPHATASE 1B (PTP1B) EXPRESSION IS INDUCED BY INFLAMMATION IN VIVO*

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Running title: PTP1B expression is induced by inflammation.

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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 proinflammatory cytokine tumor necrosis factor α (TNFα) regulate PTP1B expression in vivo. High-fat feeding of mice increased PTP1B expression 1.5-7-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 2-5-fold in a dose- and time-dependent manner in adipocyte and hepatocyte cell lines. TNFα administration to mice increased PTP1B mRNA 1.4-4-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 is a major negative regulator of insulin and leptin sensitivity, acting to dephosphorylate the insulin receptor (IR) and the leptin receptor-associated Janus kinase 2 (JAK2) (3,4). PTP1B may also dephosphorylate more distal components of these signaling pathways, such as insulin receptor substrate 1 (IRS-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, oxidation, and sumoylation have been reported to regulate PTP1B activity (4, 32), though 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 proinflammatory cytokines TNFα, interleukin-1 (IL-1), and interleukin-6 (IL-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 proinflammatory 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 IR and IRSs 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-resistant, obese, and/or diabetic 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, oxidation, and sumoylation have been reported to regulate PTP1B activity (4, 32), though 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.

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underlying tissue PTP1B overexpression in obesity and diabetes.

EXPERIMENTAL PROCEDURES

Animals- Mice and rats were housed at 22°C with a 12-hour/12-hour or 14-hour/10-hour light/dark cycle and fed ad libitum. Results are reported from random-fed mice on a standard rodent chow diet unless otherwise indicated. FVB/N 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 hour fasted ob/ob and fa/fa, and 16 hour fasted db/db mice were examined with their respective lean controls. Homozygous TNFα deficient (TNFα -/-) mice with a mixed genetic background of C57BL/6J and 129SvJ strains and wildtype F1 progeny of a C57BL/6J and 129SvJ mating were obtained from The Jackson Laboratory. TNFα -/- mice were bred to the wildtype mice to generate TNFα +/- mice. TNFα +/- mice were intercrossed to generate TNFα -/- and TNFα +/- 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, R&D664 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 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 i.p. once per day with saline or 3, 30, or 300µg dexamethasone for 4 days and sacrificed 5 hours 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 ELISA assay 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 i.v. with saline or 3.3µg of murine TNFα (Sigma) and tissues were harvested from mice 4 hours after injection. For PTP1B protein measurements, mice were injected i.v. with saline or 3.3µg murine TNFα, followed by a second intravenous injection of saline or TNFα 5 hours later. Tissues were harvested from mice 9 hours after the initial TNFα injection.

Cell culture and 3T3-L1 adipocyte differentiation- All tissue culture reagents were from Gibco (Invitrogen, Carlsbad, CA) unless otherwise indicated. HeLa cells were maintained in Dulbecco’s modified Eagle media (DMEM) with 5% fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C, 5% CO2. H-4-II-E cells were maintained in Alpha’s modification of Eagle media (αMEM)(Cellgro, Mediatech, Herndon, VA) with 5% fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C, 5% CO2. 3T3-L1 fibroblasts (obtained from the ATCC, #CCL 92.1, Rockville, MD) were grown in DMEM with 10% newborn calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C, 5% CO2. 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, St. Louis, MO) for 3 days. During and following differentiation, DMEM was supplemented with 10% fetal calf serum. Cells were used for experiments 14-21 days after induction of differentiation and only if greater than 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 dose and time indicated in figure legends. For inhibitor studies, cells were incubated with actinomycin D (2 µg/ml)(Sigma), sodium salicylate (5 mM) (Sigma), PD98059 (10 µM)(Calbiochem, San Diego, CA), SB202190 (10 µM)(Sigma), SP600125 (5 µM) (Sigma), or without inhibitor for 1-1.5 hours, followed by incubation with 20 ng/ml (1.2 nM) mouse TNFα (Sigma) or without cytokine for 4 hours.

**Immunoblotting**—Liver, perigonadal white adipose tissue, and hindlimb skeletal muscle were dissected from mice and rats and frozen immediately in liquid nitrogen. Lateral hypothalamus, medial hypothalamus (containing the ventromedial hypothalamus, VMH, and dorsomedial hypothalamus, DMH), and arcuate nucleus of the 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 Na3P2O7, 100 mM NaF, 2 mM Na3VO4, 1% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF for 1 minute with a polytron, incubated at 4°C for 40-60 minutes with rotation, and centrifuged for 10 minutes at 16,000g. 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-Aldrich, St. Louis, MO) antibodies. To detect CD68 protein in adipose tissue lysates, 400 µg of protein was incubated with wheat germ agarose overnight, 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 Corp., Piscatway, NJ) and quantified by densitometry using ImageQuant software (Molecular Dynamics/Amersham Biosciences Corp.) or direct chemiluminescence detection with GeneGnome (SynGene, Frederick, MD).

**RNA extraction and gene expression analysis by real-time RT-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 RT-PCR was performed for each sample in triplicate with 2.5 ng of total RNA, 1 X Taqman Universal Master Mix no AmpErase UNG, 6.25U of MuLV 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 sec and 60°C for 1 min. Primers and probes for 18S (which detects human and mouse 18S RNA) and suppressor of cytokine signaling 3 (SOCS-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 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.

**Chromatin immunoprecipitation (ChIP)** assays—Immunoprecipitation of NF-κB bound to PTP1B gene chromatin was accomplished using a two-step crosslinking method (47). ChIP assays were performed on 14-21 days post-differentiation 3T3-L1 adipocytes treated with...
20ng/ml (1.2nM) mouse TNFα (Sigma) or without cytokine for 4 hours. ChIP assays were additionally performed on frozen livers of random-fed 8-9 week-old female FVB mice harvested 4 hours after i.v. injection of mice with saline or 3.3μg of murine TNFα, as described above. After removal of media, adipocytes were washed 3 times with PBS prior to crosslinking. In addition, for adipocytes, all washing and crosslinking steps were carried out with the addition of 1 mM MgCl₂ to aid cell retention on plates. Cells or finely minced livers were fixed in disuccinimidyl glutarate (DSG) added to a final concentration of 2 mM in PBS for 45 minutes at room temperature, washed with 3 times with PBS, followed by fixation with 1% formaldehyde in PBS for 15 minutes at room temperature. Samples were washed 3 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 (5000X g for 5 minutes) and soluble chromatin was transferred to a new tube. Lysates were diluted in 1X RIPA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1% SDS, 140 mM NaCl, 1% triton X100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Sigma)) were precleared by incubation with 40μl of protein G agarose beads for 1 hour at room temperature, immunoprecipitated with 4μg of anti-p65 antibody (sc-109, Santa Cruz Biotechnology, Inc.) or a non-specific control antibody (CD68 antibody, Santa Cruz Biotechnology, Inc.) overnight at 4°C, and captured on protein G agarose beads for 2 hours at 4°C, all with rotation. Immunocomplexes were washed 3 times in 1X 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 hours at 65°C, extracted once with phenol-chloroform, and DNA in the aqueous phase subjected to ethanol precipitation. Chromatin resuspended in H₂O was amplified by PCR using primers 5’ CATCTCCCACGTCTTGGAAT 3’, flanking an NFκB site –892 to –883 relative to the start site of transcription of the PTP1B promoter region, predicted using MULAN software from DCODE.org Comparative Genomics Center (http://mulan.dcode.org). 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 ± SEM. 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 1-way or 2-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 (18-22) (23-28), 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 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. 1A). PTP1B expression was normal in skeletal muscle of ob/ob mice (Fig. 1A).
contrast, PTP1B protein was not overexpressed in liver, skeletal muscle, or adipose tissue of obese, insulin-resistant, type 2 diabetic db/db mice compared to lean controls (Fig. 1B) (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. 1C). Results are shown for 16-hour fasted animals, but similar differences were observed in fed ob/ob mice tissues and Zucker rat adipose tissue, and also were reported previously in studies of muscle from Zucker rats (18). We confirmed that PTP1B was overexpressed 1.5-2-fold in liver of lean streptozotocin-treated type 1 diabetic mice compared to 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 to untreated mice, indicating the development of insulin-resistance (Fig. 1D). Surprisingly, dexamethasone treatment decreased PTP1B protein levels by ~35% in liver (Fig. 1D) compared to 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 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 timepoints. At 15 weeks of age, body weight was increased by 18% (p<0.01) in mice with DIO (28.17 ± 1.23 g) compared to chow-fed controls (23.97 ± 0.74 g), but PTP1B expression was unaffected in liver or adipose tissue of mice with DIO compared to chow-fed mice (Fig. 2A). At 20 weeks of age, body weight was increased 24% (p<0.001) in mice with DIO (33.82 ± 1.28 g) compared to 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 to 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 to controls (Fig. 2B). 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. 2B). PTP1B overexpression coincided with increased expression of CD68, a macrophage marker (Fig. 2C), 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. 3A), but were increased in mice with DIO compared to chow-fed controls at 20 weeks (Fig. 3B). 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 to chow-fed mice at 20 weeks of age (Fig. 3B), but not at 15 weeks of age (Fig. 3A), consistent with the increased expression of CD68 protein observed in adipose tissue of mice with DIO at 20 weeks of age (Fig. 2C). TNFα mRNA levels were increased in adipose tissue of mice with DIO compared to chow-fed mice at 20 weeks of age (Fig. 3B), but not at 15 weeks of age (Fig. 3A), consistent with the increased expression of CD68 protein observed in adipose tissue of mice with DIO at 20 weeks of age (Fig. 2C). TNFα mRNA levels were increased in adipose tissue of mice with DIO compared to chow-fed mice at both 15 and 20 weeks, but the magnitude of increase was substantially higher in mice at 20 weeks (Fig. 3A vs. 3B). These data led us to hypothesize that tissue PTP1B overexpression in mice with DIO may be mediated by proinflammatory 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 adipose 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 3-5-fold in 3T3-L1 adipocytes in a dose and time-dependent manner (Fig. 4A-B). Similarly, TNFα treatment increased PTP1B protein levels by 2-fold in H-4-II-E hepatoma cells (Fig. 4C). TNFα also increased PTP1B protein levels by 2-3-fold in HeLa cells, in a dose- and time-dependent manner (Fig. 4D). 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 8-36 hours 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 hours (Fig. 5A). This effect was also dose-dependent (data not shown). Similarly, TNFα treatment increased PTP1B mRNA expression 2-2.5-fold in HeLa cells (Fig. 5B). 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. 5C and D). The delay in PTP1B protein overexpression compared to PTP1B mRNA upon TNFα treatment likely reflects the long half-life of PTP1B protein (≥6-8 hours)(51,52) compared to mRNA (approximately 3 hours in HeLa cells and 1.5 hours in 3T3-L1 adipocytes, Fig. 5C 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 nuclear factor kappa B (NFκB) pathway and/or one or more mitogen-activated kinases (MAPKs) including Janus-related N-terminal kinase (JNK), p38, and extracellular-related kinases 1 and 2 (ERKs) (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, 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. 6A, panel 1). Salicylate pretreatment of adipocytes also reduced TNFα-induced PTP1B mRNA expression by ~65% compared to adipocytes treated with TNFα alone (Fig. 6A, panel 1). Basal and TNFα-induced PTP1B expression were similar in adipocytes pretreated with PD98059, SB202190, or SP600125 and cells without inhibitor pretreatment (Fig. 6A, panels 2, 3, and 4). Thus, salicylate reduced the increment of TNF-α-induced PTP1B mRNA expression above basal levels in 3T3-L1 adipocytes by ~80% (Fig. 7B). In HeLa cells, treatment with SB202190 reduced the increment of TNFα-induced PTP1B mRNA expression
above basal levels by ~40% compared to 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. 7B) are similar to those seen in the expression of SOCS-3 (Fig. 7A), a gene known be regulated by TNFα. PTP1B protein was increased 1.25-fold in liver of TNFα-treated mice compared to control mice (data not shown). Because increased PTP1B mRNA precedes increased PTP1B protein by several hours in cultured cells treated with TNFα (Fig. 4 and 5), and injected TNFα is cleared within minutes to hours in mice (53,54), we injected mice twice with TNFα at 4-5 hour intervals. PTP1B protein was increased 2-fold in liver of these mice (Fig. 7C), 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 DCODE.org Comparative Genomics Center (http://mulan.dcode.org). This analysis identified a potential NFκB binding site 5’ TGGACTTTCC 3’, located –892 to –883 relative to the start site of transcription (Fig. 8A). 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 to untreated cells (Fig. 8B). 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 to untreated animals (Fig. 8C 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 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 (TNFα−/−) and wildtype (TNFα +/+ ) mice with DIO. Lean and obese TNFα−/− mice were previously reported to have increased insulin sensitivity compared to wildtype mice, due in part to enhanced insulin receptor signaling (56,57). TNFα−/− and TNFα +/+ mice on a mixed C57BL/6J X 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 TNFα -/- mice were not different from control TNFα +/- mice on either diet (Fig. 9A). Random fed blood glucose levels were similar amongst all female and amongst all male mouse cohorts (Fig. 9B). For both male and female mice, random-fed serum insulin of TNFα -/- mice was not statistically different from control TNFα +/- mice on either diet (Fig. 9C). 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 chow-fed TNFα +/- and TNFα -/- mice (Fig. 9D). Adipose tissue PTP1B expression was increased by 1.6-2.7-fold in female TNFα +/- mice with DIO compared to TNFα +/- chow-fed mice. PTP1B overexpression in TNFα -/- mice with DIO was decreased by 44-71% compared to TNFα +/- mice with DIO. Similar results were observed in adipose tissue of male mice. Adipose tissue PTP1B expression was increased by 1.7-3.1-fold in male TNFα +/- mice with DIO compared to TNFα +/- chow-fed mice (Fig. 9E). PTP1B overexpression in male TNFα -/- mice with DIO was decreased 29-100% compared to TNFα +/- mice with DIO. Due to the variability of PTP1B expression in mice of this mixed genetic background, the decreases in PTP1B expression in TNFα -/- mice were not statistically significant for all individual adipose depots of female and male mice (Fig. 9D,E) compared to TNFα +/- mice by 1-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 TNFα -/- mice was significantly lower in both female (p≤0.005) and male (p≤0.002) mice (Fig. 9D,E) compared to TNFα +/- 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 TNFα -/- 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 x 129SvJ mice with DIO. PTP1B overexpression in liver of C57Bl/6J x 129SvJ mice with DIO was higher than FVB mice with DIO, but varied up to 10 fold in both TNFα +/- and TNFα -/- 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.
resistance in skeletal muscle (58). Since diet is a key contributing factor to most forms of human obesity and since 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, since PTP1B is overexpressed in several other type 1 and type 2 diabetic animal models (data not shown)(19-21,27). 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 proinflammatory cytokines (33,59,60). Insulin, leptin, and glucose induce modest increases (1.35-2-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 effect (50) 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 to 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 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.
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 proinflammatory cytokines TNFα and IL-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 insulin 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 proinflammatory cytokines have been proposed to be a causal link between obesity and type 2 diabetes (33,34). Thus, increased expression of TNFα or other proinflammatory 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 types 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 types of insulin-resistance (i.e. those caused by inflammation) in which PTP1B overexpression may play a causal role.

Whereas 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.
REFERENCES


FOOTNOTES

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**FIGURE LEGENDS**

**FIG. 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 8-10-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 ± SEM (n=4-7 per group). * p≤0.05 compared to 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 ± SEM (n=3 per group). * p≤0.05 compared to untreated mice by 1-way ANOVA. (E). PTP1B protein levels in liver, skeletal muscle, and perigonadal white adipose tissue (WAT) of dexamethasone-treated mice were determined by immunoblotting. Results are means ± SEM (n=3 per group). * p≤0.05 compared to control or untreated mice by 1-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.

**FIG. 2.** Overexpression of PTP1B protein in insulin-target tissues and the arcuate nucleus of hypothalamus of mice with diet-induced obesity (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 ± SEM (n=8-9 per group). (B). PTP1B protein levels in liver, skeletal muscle, perigonadal white adipose tissue (WAT), arcuate nucleus of the 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 ± SEM (n=7-8 per group). * p≤0.05 compared to 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.

**FIG. 3.** Increased TNFα expression in adipose tissue accompanies PTP1B overexpression in insulin- and leptin-target tissues of mice with diet-induced obesity (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 RT-PCR. Results are means ± SEM (n=7-9 per group). * p≤0.05 compared to chow-fed animals by T-test.

**FIG. 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 concentration of TNFα for 16 hours (A, top panel and B, left panel) or with 1nM TNFα for the indicated times (A, bottom panel and B, right panel). Representative immunoblots for PTP1B (A) and means ± SEM (n=2-3 per condition) (B) are shown. (C). PTP1B protein from H-4-II-E hepatoma cells incubated with the indicated concentration of TNFα for 16 hours. Results are means ± SEM (n=3 per condition). (D). PTP1B protein from HeLa cells incubated with the indicated concentration of TNFα for...
15-16 hours (left) or with 3nM TNFα for the indicated time (right). Results are means ± SEM (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≤0.05 compared to cells without TNFα by 1-way ANOVA.

**FIG. 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 0.2nM TNFα for the indicated time is shown. Results are means ± SEM (n=2 per condition). (B). PTP1B mRNA from HeLa cells incubated with the indicated concentration of TNFα for 15 hours (left) or with 3nM TNFα for the indicated time (right) is shown. Results are means ± SEM (n=3 per condition). * p≤0.05 and & p≤0.1 compared to untreated cells by 1-way ANOVA. (C-D). PTP1B mRNA from 14-21-day post-differentiation 3T3-L1 adipocytes (C) or HeLa cells (D) treated with actinomycin D (aD) (2µg/ml) or without inhibitor for 1 hour before incubation with 1.2nM TNFα or without cytokine for 4 hours. Results are means ± SEM (n=6 in C and n=12 in D, per condition). * p≤0.05 compared to the corresponding condition without TNFα, and # p≤0.05 compared to the corresponding condition without actinomycin D by 2-way ANOVA. For all experiments, PTP1B mRNA and control 18S rRNA were measured by real-time quantitative RT-PCR and PTP1B mRNA was normalized to 18S rRNA.

**FIG. 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 (5mM), PD98059 (10µM), SB202190 (10µM), SP600125 (5µM), or without inhibitor for 1 hour, followed by incubation with 1.2nM TNFα or without cytokine for 4 hours is shown. Results are means ± SEM (n=6 per condition). * p≤0.05 compared to the corresponding condition without TNFα and # p≤0.05 compared to the corresponding condition without inhibitor by 1-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.

**FIG. 7.** Acute TNFα treatment increases PTP1B expression in insulin- and leptin-target tissues of mice.

(A-B). FVB mice were injected i.v. with saline or TNFα (3.3µg per mouse) and sacrificed 4 hours later. PTP1B mRNA, SOCS-3 mRNA, and 18S rRNA from liver, gastrocnemius skeletal muscle, perigonadal adipose tissue (WAT), and the arcuate nucleus of the hypothalamus were measured by real-time quantitative PCR. SOCS-3 (A) or PTP1B (B) mRNA was normalized to 18S rRNA. Results are means ± SEM (n=6 per condition). (C). FVB mice were injected i.v. with saline or 3.3µg TNFα, followed by a second intravenous injection of the same 5 hours later. Tissue was harvested from mice 9.5 hours 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 ± SEM (n=6 per condition). * p≤0.05 compared to untreated mice by T-test.

**FIG. 8.** TNFα induces NFκB subunit p65 binding to the mouse PTP1B promoter in 3T3-L1 adipocytes in vitro and mouse liver in vivo.

(A). 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 treated with saline (basal) or TNFα for 4 hours (C). Chromatin was immunoprecipitated (IP) with antibodies specific for the NFκB subunit p65 or control (ctl) antibodies. 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 ± SEM of immunoprecipitated PCR products normalized to input PCR products for each sample (n=4 per group). *p≤0.05 compared to saline-treated mice.

FIG. 9. TNFα deficiency blunts PTP1B overexpression in adipose tissue of mice with diet-induced obesity (DIO).
TNFα+/+ and TNFα−/− 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 ± SEM (n=6-8 per group). * p≤0.05 compared to WT chow-fed mice by 1-way ANOVA. (D,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 ± SEM 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≤0.05 compared to WT chow-fed mice and # p≤0.05 compared to WT DIO mice. Symbols above bars indicate differences determined by 1-way ANOVA. Symbols above lines indicate differences determined by 2-way ANOVA, with mouse group and adipose depot being the two factors.
Figure 1

a. PTP1B protein (AU)

b. PTP1B protein (AU)

c. PTP1B protein (AU)

d. Serum insulin (ng/ml)

e. PTP1B protein (AU)
Figure 2

(a) Liver and perigonadal white adipose tissue (WAT) PTP1B protein levels in chow-fed and diet-induced obesity (DIO) mice.

(b) PTP1B protein levels in liver, skeletal muscle, and perigonadal WAT comparing chow-fed and DIO mice.

(c) Representative blots showing CD68 expression in diet-induced obesity (DIO) and chow-fed mice.
Figure 3
Figure 4

a. 3T3-L1 adipocytes

16 hours TNFα:

0 0.04 nM 0.2 nM 1 nM 5 nM

PTP1B protein (AU)

b. 3T3-L1 adipocytes

nM TNFα for 16 hr

0 0.04 0.2 1 5

hours of 1nM TNFα

0 4 7 17 24 36

---

c. H-4-II-E hepatocytes

PTP1B protein (AU)

0 100 200 300

nM TNFα for 16 hr

0 0.12 0.6 3

---

d. HeLa cells

PTP1B protein (AU)

0 100 200 300

nM TNFα for 16 hr

0 0.04 0.2 1 5

hours of 3nM TNFα

0 4 8 12 16 26
Figure 5

a. 3T3-L1 adipocytes

PTP1B mRNA (AU)

0 4 8 16 24

hours of 0.2nM TNFα

b. HeLa cells

nM TNFα for 15 hr

0 .02 .12 .6 3 12

c. 3T3-L1 adipocytes

hours of 3nM TNFα

0 1 2 4 8 12

d. HeLa cells

- - aD aD

- - aD aD

- - aD aD
Figure 6

**Figure 6**

**a.** PTP1B mRNA (AU) in 3T3-L1 adipocytes with/without inhibitor stimulation.

**b.** PTP1B stimulation with/without inhibitor in 3T3-L1 adipocytes.
Figure 7

a. SOCS-3 mRNA (AU)

b. PTP1B mRNA (AU)

c. PTP1B protein (AU)
Figure 8

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3T3-L1 adipocytes

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c.  

Liver

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<th>Primers</th>
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<td>-933 to -626</td>
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<tr>
<td>IP ctl Ab</td>
<td>-933 to -626</td>
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<tr>
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<td>IP ctl Ab</td>
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d.  

% of basal IP p65 for each PCR product

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</table>

* Significant difference
Figure 9

**a.** female vs. male weight (g) comparisons.

**b.** female vs. male blood glucose (mg/dl) comparisons.

**c.** female vs. male serum insulin (ng/ml) comparisons.

**d.** female vs. male PTP1B protein (AU) comparisons.

**e.** male vs. male PTP1B protein (AU) comparisons.
Protein tyrosine phosphatase 1B (PTP1B) expression is induced by inflammation in vivo
Janice M. Zabolotny, Young-Bum Kim, Laura A. Welsh, Erin E. Kershaw, Benjamin G. Neel and Barbara B. Kahn

J. Biol. Chem. published online February 14, 2008

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