Deletion of Protein Tyrosine Phosphatase 1b Improves Peripheral Insulin Resistance and Vascular Function in Obese, Leptin-Resistant Mice via Reduced Oxidant Tone

M. Irfan Ali,* Pimonrat Ketsawatsomkron,* Eric J. Belin de Chantemele, James D. Mintz, Kenjiro Muta, Christina Salet, Stephen M. Black, Michel L. Tremblay, David J. Fulton, Mario B. Marrero,† David W. Stepp†

Rationale: Obesity is a risk factor for cardiovascular dysfunction, yet the underlying factors driving this impaired function remain poorly understood. Insulin resistance is a common pathology in obese patients and has been shown to impair vascular function. Whether insulin resistance or obesity, itself, is causal remains unclear.

Objective: The present study tested the hypothesis that insulin resistance is the underlying mediator for impaired NO-mediated dilation in obesity by genetic deletion of the insulin-desensitizing enzyme protein tyrosine phosphatase (PTP)1B in db/db mice.

Methods and Results: The db/db mouse is morbidly obese, insulin-resistant, and has tissue-specific elevation in PTP1B expression compared to lean controls. In db/db mice, PTP1B deletion improved glucose clearance, dyslipidemia, and insulin receptor signaling in muscle and fat. Hepatic insulin signaling in db/db mice was not improved by deletion of PTP1B, indicating specific amelioration of peripheral insulin resistance. Additionally, obese mice demonstrate an impaired endothelium dependent and independent vasodilation to acetylcholine and sodium nitroprusside, respectively. This impairment, which correlated with increased superoxide in the db/db mice, was corrected by superoxide scavenging. Increased superoxide production was associated with increased expression of NAD(P)H oxidase 1 and its molecular regulators, Noxo1 and Noxa1.

Conclusions: Deletion of PTP1B improved both endothelium dependent and independent NO-mediated dilation and reduced superoxide generation in db/db mice. PTP1B deletion did not affect any vascular function in lean mice. Taken together, these data reveal a role for peripheral insulin resistance as the mediator of vascular dysfunction in obesity. (Circ Res. 2009;105:1013-1022.)

Key Words: obesity ■ leptin resistance ■ PTP1B

The prevalence of obesity and its cardiovascular complications represents a significant health concern in Western societies, but the root causes of cardiovascular dysfunction in obese individuals remain unclear. Metabolic dysfunction, notably insulin resistance, is evident in obesity. It has been speculated that insulin resistance, rather than other aspects of obesity, is the underlying cause of cardiovascular injury in obese patients. This hypothesis has been difficult to test because insulin-sensitizing drugs have off-target effects and nonobese models of insulin resistance do not evaluate the relative importance of obesity versus insulin resistance.

The insulin receptor is a classic receptor tyrosine kinase and, as such, is deactivated by protein tyrosine phosphatases, notably protein tyrosine phosphatase (PTP)1B. Deletion of PTP1B improves insulin sensitivity in mouse models of obesity, and putative PTP1B antagonists have been used pharmacologically to improve glucose tolerance. Increases in the activity and/or expression of PTP1B correlate with blunted insulin signaling in a variety of tissue types. Whether PTP1B deletion and amelioration of insulin resistance improves cardiovascular dysfunction associated with obesity remains unknown.

The present study tested the hypothesis that PTP1B deletion attenuates vascular dysfunction in a model of obesity-induced insulin resistance. Four experimental genotypes were generated through breeding of db/db and PTP1B−/− mice.
to produce double knockout (KO) PTP1B-null, obese mice. Metabolic profiling, insulin receptor phosphorylation, and PTP1B gene expression were used to assess insulin sensitivity in target tissues. Endothelium-dependent and -independent vascular function were determined in vitro. Molecular techniques examined the mechanism by which deletion of PTP1B improved vascular function. Taken together, these studies critically test the hypothesis that insulin resistance in obesity is the underlying risk factor driving vascular dysfunction in obese individuals.

Methods

Animal Models

All mice used are held in the AAALAC-accredited Medical College of Georgia animal housing facilities. The animals are housed in clean cages with adequate food and water. The Medical College of Georgia IACUC monitors all animal usage on campus. All of our experimental protocols have been institutionally approved.

Two parental strains of mice were used in these studies: leptin receptor mutant db/db mice bred on a C57BL/6 background (The Jackson Laboratories) and PTP1B-null mice bred on a BALB/c background (Michel Tremblay, PhD, Cancer Institute of McGill University). Because db/db mice are sterile, progeny were generated in ovariectomized (OVX) Hsd:NMRI CB6-1LeprΔob/J females crossbred to Hsd:NMRI CB6-1LeprΔob/J males (Jackson Laboratories) and PTP1B-null mice bred on a BALB/c background (The Jackson Laboratories) for 1:4 and 1:8 ratios, respectively. Dual heterozygous heterozygous for mutant leptin receptor and PTP1B gene deletion. Dual heterozygotes were interbred, producing obese, PTP1B gene–null, and dual KO mice at 1:4, 1:4, and 1:16 ratios, respectively. In the F1 generation, dual heterozygotes were bred to heterozygotes for the leptin receptor mutation and PTP1B gene–null mice. This breeding strategy yielded obese and dual KO mice at 1:4 and 1:8 ratios, respectively. Dual heterozygous littersmates were used as lean controls, and littersmates heterozygous for db and PTP1B gene deletion were used as lean PTP1B-null controls. All experiments were conducted in male progeny. In all cases, mice are designated as H or K, indicating heterozygote or KO. The db gene is designated first and the PTP1B second. Thus, Hdb/HPTP, are heterozygous for both genes, Hdb/KPTP are lean PTP1B KO mice, Kdb/HPTP are obese mice with intact PTP1B, and Kdb/KPTP are deficient in both leptin receptors and PTP1B. Mice were genotyped by PCR of genomic DNA. Metabolic phenotyping was accomplished by assessment of glucose tolerance and plasma chemistry (Online Data Supplement, available at http://circres.ahajournals.org).

Insulin Signaling

To determine the effects of PTP1B deletion on insulin receptor phosphorylation, mice were subjected to an insulin stimulation protocol in vivo. Briefly, mice were anesthetized with isoflurane, and either saline or insulin (1 mU/g) was injected into a jugular vein catheter. After 12 minutes, mice were euthanized with isoflurane overdose and samples of liver, skeletal muscle, and adipose tissue were obtained and snap-frozen in liquid nitrogen. The time period between overdose and tissue harvesting was less than 2 minutes total and samples were obtained in differing order to avoid collection bias.

Western Blotting

Tissue homogenates (20 to 50 μg) were separated via SDS-PAGE and transferred to Immobilon-P poly(vinylidene fluoride) membranes. To determine the expression of relevant proteins, immunoblots were probed with antibodies for PTP1B (Upstate), actin (Calbiochem), insulin receptor-β (Santa Cruz Biotechnology), endothelial NO synthase, and phosphorylated endothelial NO synthase 117/79 (BD Transduction Laboratories).

Insulin Receptor Phosphorylation

An anti–insulin receptor-β antibody was used to immunoprecipitate insulin receptor proteins from 400 to 1000 μg of tissue lysates, and phosphorylation status was assessed with phospho-tyrosine antibody (PY4G10, Upstate) (Online Data Supplement).

In Vitro Microvessel Preparation

Small mesenteric arteries (SMAs) are defined in this study as the conduit superior mesenteric artery ranging from 50 to 150 μm in length. SMAs were dissected and segments (0.25 to 1 mm in length) were mounted in a vessel bath between two glass micropipettes (25 μm-diameter tip) and secured with 10-0 silk ophthalmic suture. SMAs were then placed in a chilled, oxygenated (21% O2, 5% CO2, and 74% N2) Krebs–Ringer bicarbonate solution composed of (in mmol/L) 118.3 NaCl, 4.7 KCl, 1.2 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11.1 d-glucose before analysis to hibernate physiological activity. The lumen of the vessel was filled with Krebs buffer through the micropipette and maintained at a constant pressure of 60 mm Hg. Vessels were monitored under a Nikon inverted light microscope (Melville, NY) connected to a video monitor. Internal diameter was continually measured using video calipers and expressed in micrometers. Buffer temperature was increased to 37°C, and microvessels were allowed to develop spontaneous myogenic tone. After tone was developed, vasodilator responses were measured with sequential doses of acetylcholine (1 × 10-10 to 1 × 10-5 mol/L), sodium nitroprusside (SNP) (1 × 10-9 to 1 × 10-4 mol/L), or papaverine (1 × 10-9 to 1 × 10-4 mol/L). Superoxide dismutase (SOD) was used to scavenge superoxide (100 U/mL), and Samples of liver, skeletal muscle, and adipose tissue were obtained and snap-frozen in liquid nitrogen. Total RNA was extracted using TRIzol Plus RNA (Invitrogen), and cDNA was synthesized using the iScript cDNA Synthesis Kit (BioRad). Real-Time RT-PCR

Mesenteric arterial cascades were harvested from euthanized animals, removed of nonvascular tissue, and snap-frozen in liquid nitrogen. The quantitative abundance of superoxide was assessed using electron paramagnetic resonance (EPR) spectroscopy. Qualitative assessment of superoxide localization was made using dihydroethidium staining (Online Data Supplement).
Kit (Bio-Rad). cDNA was then used to assess relative gene expression using real-time RT-PCR (Bio-Rad iQ SYBR Green). Primer sequences for the selected genes are described in Online Table I.

Statistics

All data are expressed as means±SEM. Differences among all 4 genotypes were compared by 1-way ANOVA or by Student t test with Bonferroni correction test used as the post hoc test. A probability value of less than 0.05 was considered statistically significant.

Results

Weight Gain

Increases in body mass are depicted in Online Figure II. Data are presented as a scatter plot of 5 male mice of each genotype. Summary data at age 12 weeks are shown in the Table. Consistent with the inactivating mutation in the leptin receptor gene, K<sub>db</sub>H<sub>PTP</sub> mice displayed morbid obesity compared to H<sub>db</sub>H<sub>PTP</sub> mice. Deletion of PTP1B did not affect weight gain in either H<sub>db</sub>K<sub>PTP</sub> or K<sub>db</sub>K<sub>PTP</sub> mice compared to PTP1B intact controls. H<sub>db</sub>K<sub>PTP</sub> mice had modest reductions in plasma leptin versus H<sub>db</sub>H<sub>PTP</sub> mice, consistent with sensitization of the leptin receptor, which is also a substrate of PTP1B. In db/db mice, plasma leptin levels were markedly increased along with body weight and were unaffected by deletion of PTP1B (Table).

Food and water intake and urine output are also summarized in the Table. H<sub>db</sub>H<sub>PTP</sub> and H<sub>db</sub>K<sub>PTP</sub> mice exhibited normal and similar food and water intake and urine output. K<sub>db</sub>H<sub>PTP</sub> mice displayed hyperphagia, polydypsia, and polyuria, consistent with obesity. Deletion of PTP1B on the obese background did not affect food and water intake.

In summary, these data indicate that the fundamental defects in leptin signaling that drive obesity in db/db mice are not moderated by the deletion of PTP1B. Thus, the metabolic improvements arising from PTP1B deletion must be attributed to changes in insulin receptor signaling and not modification of obesity.

Glucose Metabolism

Baseline levels of plasma glucose and insulin are shown in the Table. Fasting glucose in H<sub>db</sub>H<sub>PTP</sub> mice was euglycemic and deletion of PTP1B in H<sub>db</sub>K<sub>PTP</sub> mice did not alter fasting glucose. The obesity observed in K<sub>db</sub>H<sub>PTP</sub> mice was associated with moderate hyperglycemia. Deletion of PTP1B in K<sub>db</sub>K<sub>PTP</sub> mice did not reduce fasting blood glucose, suggesting persistent hepatic insulin resistance in the K<sub>db</sub>K<sub>PTP</sub> mice. Consistent with these observations, H<sub>db</sub>H<sub>PTP</sub> mice were eusulinemic but K<sub>db</sub>H<sub>PTP</sub> and K<sub>db</sub>K<sub>PTP</sub> mice had persistent hyperinsulinemia. Insulin levels in H<sub>db</sub>K<sub>PTP</sub> mice were similar to H<sub>db</sub>H<sub>PTP</sub> mice.

In vivo clearance of a glucose bolus is shown in Online Figure III (S3). H<sub>db</sub>H<sub>PTP</sub> mice displayed rapid glucose disposal, and clearance of glucose in H<sub>db</sub>K<sub>PTP</sub> mice was similar. K<sub>db</sub>K<sub>PTP</sub> mice showed markedly blunted glucose clearance, but K<sub>db</sub>K<sub>PTP</sub> mice showed normalization of glucose clearance despite obesity.

Hemoglobin (Hb)A<sub>1c</sub> levels, an index of total glycemic load, are shown in the Table. H<sub>db</sub>H<sub>PTP</sub> and H<sub>db</sub>K<sub>PTP</sub> mice showed HbA<sub>1c</sub> levels lower than 5%, consistent with euglycemic control. In contrast, K<sub>db</sub>H<sub>PTP</sub> mice showed markedly elevated HbA<sub>1c</sub> levels, consistent with their observed glucose intolerance and fasting hyperglycemia. Although not completely normalized, HbA<sub>1c</sub> levels were significantly reduced in K<sub>db</sub>K<sub>PTP</sub> mice compared to K<sub>db</sub>H<sub>PTP</sub> mice, despite equivalent food intake.

Lipid Metabolism

Plasma concentration of free fatty acids (FFAs), triglycerides, and cholesterol are shown in the Table. H<sub>db</sub>H<sub>PTP</sub> and H<sub>db</sub>K<sub>PTP</sub> mice show normal levels of all 3 lipid compounds. K<sub>db</sub>H<sub>PTP</sub> mice had elevated fasting FFAs and increased triglyceride levels, consistent with the loss of insulin sensitivity in fat cells. K<sub>db</sub>K<sub>PTP</sub> mice displayed markedly reduced FFAs and lower triglycerides, suggesting a normalization of adipocyte insulin resistance by deletion of PTP1B. In contrast, total cholesterol was elevated to a similar extent in both K<sub>db</sub>H<sub>PTP</sub> and K<sub>db</sub>K<sub>PTP</sub> mice.

Expression of PTP1B

To determine the effects of obesity on the tissue expression of PTP1B, Western blotting was performed on extracts from liver, muscle, and fat, which are the 3 major targets of the metabolic actions of insulin. The results are shown in Figure 1A through 1C. PTP1B expression was heterogeneous with marked increases in expression in the skeletal muscle (Figure 1B) and adipose tissue (Figure 1C) of obese mice. Expression of PTP1B in the liver (Figure 1A) was not statistically different between lean H<sub>db</sub>H<sub>PTP</sub> and obese K<sub>db</sub>H<sub>PTP</sub> mice.
Insulin Signaling

Phosphorylation of the insulin receptor was used as a molecular readout of insulin signaling capacity. In skeletal muscle, adipose, and liver tissue samples, insulin provoked a marked increase in receptor tyrosine phosphorylation that was similar in \( H_{db}H_{PTP} \) and \( H_{db}K_{PTP} \) mice. In contrast, in \( K_{db}K_{PTP} \) mice, the tyrosine phosphorylation of the insulin receptor was markedly reduced, consistent with obesity-induced insulin resistance. In skeletal muscle (Figure 1E) and adipose tissue (Figure 1F), insulin receptor phosphorylation was markedly increased in \( K_{db}K_{PTP} \) mice, suggesting that deletion of PTP1B improved insulin signaling. In contrast, insulin receptor phosphorylation remained depressed in the liver (Figure 1D) of \( K_{db}K_{PTP} \) mice, suggesting persistent hepatic insulin insensitivity in these animals.

**Figure 1.** The PTP1B gene was significantly upregulated in obese, insulin-resistant mice compared to control in muscle (B) and fat (C) but not hepatic tissues (A). PTP1B gene deletion markedly improves insulin receptor tyrosine phosphorylation in muscle (E) and fat (F) of dual KO animals compared to obese, insulin-resistant animals. Dual KO hepatic tissue insulin resistance persisted (D). Control animals and PTP1B KO lean animals displayed similar in insulin receptor signaling in all tissues. *\( P < 0.05 \) vs \( H_{db}H_{PTP} \) (n>5).
Vascular Reactivity

Endothelium-dependent, acetylcholine-mediated vasodilation in the SMAs from all mice is shown in Figure 2A. Smooth muscle reactivity to NO was determined using SNP (Figure 2B). Lean mice that are PTP1B-deficient (H\textsubscript{db}K\textsubscript{PTP}) do not have differences in maximum dilation to acetylcholine (70% versus 68%, \( P = \text{NS} \)) or SNP (96% versus 94%, \( P = \text{NS} \)) from H\textsubscript{db}H\textsubscript{PTP} mice, indicating that PTP1B deletion does not affect endothelium-dependent or -independent vasodilation. The maximum vasodilator response to acetylcholine was reduced markedly in K\textsubscript{db}H\textsubscript{PTP} compared to H\textsubscript{db}H\textsubscript{PTP} (50% versus 70%, \( P < 0.05 \)), indicating impairment of endothelial function in K\textsubscript{db}H\textsubscript{PTP} mice. A significant deficiency in reactivity to exogenous NO was also detected in K\textsubscript{db}H\textsubscript{PTP} mice (68% versus 94%, \( P < 0.05 \)). The maximum vasodilator response to acetylcholine was markedly reduced in all mice following treatment with 100 \( \mu \)mol/L L-NAME (Figure 2C), indicating NO as the primary dilator mediating the response to acetylcholine. Furthermore, the NO-independent component of acetylcholine-induced vasodilation was not different among all groups of mice. Taken together, these data indicate that vasodilator reactivity is compromised at the level of NO utilization in obese mice. In contrast to the findings observed in K\textsubscript{db}H\textsubscript{PTP} mice, endothelium-dependent vasodilation in obese K\textsubscript{db}K\textsubscript{PTP} was similar to that observed in lean H\textsubscript{db}H\textsubscript{PTP} mice (Figure 2A). The impaired response to exogenous NO (SNP) was also restored by PTP1B deletion (Figure 2B). Vascular dysfunction was not attributable to loss of endothelial NO synthase expression or phosphorylation because these variables were similar in all strains of mice (Online Figure IV; S4). Responses to the NO-independent vasodilator papaverine were similar across all mice (Online Figure V; S5), suggesting that the vasodilation deficiency in K\textsubscript{db}H\textsubscript{PTP}
mice is not attributable to a general deficit in vascular dilation but is confined to NO-mediated dilation.

To determine whether elevated superoxide production was a mechanism of impaired vasodilation in obesity, vascular function was assessed in the presence of 100 U/mL pegylated (PEG)-SOD. PEG-SOD reversed the impaired dilation to acetylcholine (Figure 2D) and SNP (Figure 2E) in K/dbH_PTP mice, with no effect on vascular function in the other genotypes. To further determine whether PEG-SOD was indeed restoring NO bioactivity, endothelium-dependent dilation was assessed in the presence of PEG-SOD and both the presence and absence of 100 μmol/L L-NAME (Online Figure VI; S6). All mouse vessels exhibited equivalent degrees of L-NAME–resistant dilation, thus confirming that the main dilation mechanism in these microvessels is NO and further that scavenging of superoxide did not improve NO-independent dilation in K/dbH_PTP mice.

Passive mechanics were assessed in a zero-Ca²⁺ Krebs solution and results are shown in Online Table II (ST2). Vascular architectural changes were assessed as previously described. Maximal vessel wall thickness and wall to lumen ratio (at 120 mm Hg of intraluminal pressure) were similar across all genotypes. Vascular compliance, as calculated by the exponential fit of a circumferential stress-strain plot (β-coefficient), also remained similar across all genotypes. Taken together, these data indicate that neither obesity nor deletion of PTP1B produce structural changes that could account for observed deficits in vasodilator function.

**Superoxide Production**

EPR spectroscopy was used to semiquantitatively measure superoxide. The relative PEG-SOD–inhibitable signal was 4 times higher in mesenteric vessels from K/dbH_PTP mice versus
SOD2 and SOD3 in K
db/db mice was nearly eliminated following acetovanillone (apocynin) incubation (Figure 3B), suggesting that it derives from NAD(P)H oxides. Taken together, peripheral insulin resistance increases vascular superoxide production that is corrected by PTP1B deletion. As a further measure of vascular reactive oxygen species production, we also performed dihydroethidium staining of blood vessels. These results are in agreement with the EPR studies and demonstrate higher levels of superoxide in the blood vessels of K
db/db mice compared to controls (Figure 3C). The deletion of PTP1B in obese animals (K
db/db) decreased dihydroethidium staining to control levels, and there was no difference in superoxide production resulting from the deletion of PTP1B in lean animals. Dihydroethidium staining was most intense in the medial layer of mesenteric microvessels in all 4 groups of mice.

The source of elevated superoxide levels in K
db/db mice (Figure 3A) and reversed to control levels by deletion of PTP1B in obese K
db/db mice showed major increases in Nox1, Noxα1, and Noxα1 gene expression. *P<0.05 all genotypes vs H
ob/H
ob; †P<0.05 K
ob/K
ob vs K
ob/H
ob (n>6).

Figure 4. Gene expression of superoxide-generating and antioxidant defense enzymes were assessed using quantitative real-time RT-PCR. K
ob/H
ob mice showed major increases in Nox1, Noxα1, and Noxα1 gene expression. *P<0.05 all genotypes vs H
ob/H
ob (n>6); †P<0.05 K
ob/K
ob vs K
ob/H
ob (n>6).

Discussion
The goal of the present study was to determine whether the metabolic consequences of obesity or the state of obesity itself results in vascular dysfunction. To test this hypothesis, we generated obese mice harboring deletion of PTP1B, a tyrosine phosphatase that antagonizes insulin signaling. The key findings of this study are as follows: (1) deletion of PTP1B in db/db mice does not affect obesity and corrects peripheral but not hepatic insulin resistance; (2) correction of peripheral insulin resistance improves NO-mediated dilation in SMAs despite persistent obesity; and (3) correcting peripheral insulin resistance mediates the improvement in NO dilation by decreasing superoxide levels, primarily through reduced expression of Nox1, Noxα1, and Noxα1.

Metabolic Effects of PTP1B Deletion in Obese Mice
Although PTP1B has attracted considerable attention as a target in the treatment of non–insulin-dependent diabetes, the impact of obesity on the relative distribution of PTP1B expression in tissues central to insulin action is unclear. Moreover, expression of PTP1B in models of obesity and diabetes is complicated because it varies with the stage of diabetes and genetic background.30 As shown in Figure 1A through 1C, obesity in the db/db mice used in these studies caused a differential increase in PTP1B expression, with the most prominent increases in muscle and fat and a statistically undetectable difference in the liver. The increases in PTP1B expression correlate with decreased insulin receptor phosphorylation and are reversed by PTP1B deletion.

Deletion of PTP1B did not affect weight gain in either lean or obese mice. Food and fluid intake, urine output, and plasma leptin levels also remained unchanged. These observations are consistent with those of Cheng et al.,31 in which ob/ob mice heterozygous for PTP1B and ob/ob mice with deletion PTP1B showed similarity in weight gain. In contrast, adenosvirial delivery of PTP1B antisense RNA produces reductions in body weight and fat mass,32 and when ob/ob mice that lack PTP1B are compared to ob/ob mice with wild-type PTP1B expression, an ≈15% weight difference is observed.31 It is important to note that in this study, deletion of PTP1B improves glycemic control in ob/ob mice when wild-type (2 copies) and KO (no copies) are compared. However, one could not determine in this setting whether the improvement in glycemic control reflects the actions of PTP1B on insulin signaling or the weight loss in ob/ob mice. In the present study, we evaluated metabolic control between mice in which body weight is identical and the leptin receptor is completely missing. Thus, differences between K
ob/H
ob and K
ob/K
ob, which are equally obese, reflect the effects of PTP1B deletion on improvements in the insulin signaling pathway.

To verify the functional importance of the increase in PTP1B expression in K
ob/H
ob mice, we used physiological (plasma serum chemistry and glucose tolerance) and molecular (phosphorylation of the insulin receptor) measurements as indices of insulin signaling. Consistent with recent observations from Delibegovic et al.,33 the elevated expression of PTP1B in skeletal muscle of obese mice correlated with marked impairment in muscle insulin receptor phosphorylation and significant impairment in glucose tolerance and increased HbA1c percentage. These variables were markedly improved by the deletion of PTP1B. These findings, combined with the marked improvement in insulin receptor...
tyrosine phosphorylation in muscle indicate that PTP1B is a key determinant of skeletal muscle insulin sensitivity in db/db mice.

Reductions in serum triglycerides and FFAs following deletion of PTP1B, combined with the marked increase in PTP1B expression in visceral adipose tissue and improvement in insulin receptor signaling in visceral adipose tissue, indicate that PTP1B also plays a critical role in the adipose tissue of obese mice. In this study, the lack of a leptin receptor results in similar body weight between K_{db}H_{PTP} and K_{db}K_{PTP} mice but marked differences in triglyceride and FFA levels in the plasma, indicating an improvement of insulin signaling in fat tissue. Plasma cholesterol was elevated in K_{db}H_{PTP} mice compared to H_{db}H_{PTP} mice (Table) but was not affected by PTP1B deletion in H_{db}K_{PTP} or K_{db}K_{PTP} mice. Because both K_{db}H_{PTP} and K_{db}K_{PTP} mice retain the hyperphagic phenotype, the lack of a difference in plasma cholesterol likely indicates that elevated cholesterol in these animals reflects dietary intake or persistent hepatic insulin resistance.

Fasting blood glucose, primarily driven by hepatic gluconeogenesis, was similar in K_{db}H_{PTP} and K_{db}K_{PTP} mice. Plasma insulin levels are also elevated, consistent with the loss of insulin receptor function in the liver.4,34,35 PTP1B expression was not significantly increased in the liver and insulin receptor phosphorylation remains depressed in K_{db}K_{PTP} mice despite deletion of PTP1B. Although previous studies have described a role for PTP1B in hepatic insulin signaling in lean mice36,37 or with nongenetic methods,38 our model does not reflect this outcome, likely because of the background of these mice.30 Nevertheless, the lack of improvement in hepatic insulin signaling and moderate fasting hyperglycemia indicate that hepatic insulin resistance cannot explain observed impairments in cardiovascular function.

Effect of Deletion of PTP1B on Vasodilation

Previous studies in obese rodents have indicated that obesity is a risk factor for vascular dysfunction,11,39,40 but the culpable component of obesity has remained elusive. As described above, the dual KO mice developed for these studies remain obese, but peripheral insulin resistance is improved when PTP1B is deleted. Moreover, the results described in these studies indicate that when PTP1B is deleted in obese mice, vasodilation to NO is improved.

The role of PTP1B in improving NO-mediated vasodilation could be attributed to (1) a direct effect of PTP1B on endothelial function or (2) an improvement in vasodilation secondary to correction of peripheral insulin resistance. A direct effect of PTP1B deletion is refuted by the lack of vascular outcomes in lean H_{db}K_{PTP} mice, consistent with previous work in which overexpression of PTP1B in cultured endothelial cells did not influence the function of endothelial NO synthase.41 These observations preclude PTP1B as a direct modulator of vasodilation.

Our observations are more consistent with the hypothesis that insulin resistance is the causal factor in vessel dysfunction in obesity. To date, this hypothesis has been primarily based on studies in nonobese models of insulin resistance11,13,42,43 and studies with pharmacological compounds.4,10,42 Clear interpretation of these studies is confounded by off-target effects of drugs and because they lack the physiological context of obesity. In the present study, we have developed a novel double KO model and characterized in detail the metabolic parameters relevant to insulin resistance. The outcome is that we have described a model in which improvement of peripheral insulin resistance improves vascular function, despite persistent obesity and modest hyperglycemia. This normalization provides strong evidence that obesity has minimal impact on vasodilation mediated by NO in the absence of insulin resistance. Because the deletion of PTP1B in this model improves peripheral but not hepatic insulin resistance, we can refine the metabolic hypothesis of vessel dysfunction beyond whole body insulin resistance to specific compartments. Impairment of vascular function correlates with markers of metabolic dysfunction in muscle and fat but not the liver. To our knowledge, this is the first study to localize vascular dysfunction in obesity to peripheral insulin resistance.

Effect of PTP1B Deletion on Reactive Oxygen Species

The mechanisms by which insulin resistance impairs NO-mediated vasodilation are incompletely understood. In the present study, we present evidence that the primary mechanism is an increase in reactive oxygen species. In obese mice, the level of superoxide is increased versus lean controls (Figure 3A and 3C); vasodilation is restored by oxidant scavenging and components of the NAD(P)H oxidase pathway are increased (Figure 2D and 2E; Figure 4; Online Table III; ST3). Blockade of NAD(P)H oxidases normalizes oxidant load. Correction of insulin resistance by deletion of PTP1B in obese mice corrects the augmented reactive oxygen species levels. Scavenging of superoxide restores sensitivity to acetylecholine as does deletion of PTP1B in K_{db}K_{PTP} mice. The L-NAME−resistant component of endothelium-dependent dilation remains the same in all mice, ruling out differences in NO-independent endothelial vasodilation. Taken together, these data suggest that insulin resistance corrupts endothelial vasodilation by NAD(P)H oxidase–derived oxidants.

Previous studies have attributed increases in superoxide levels to an increase in NAD(P)H oxidase activity.44,45 Nox2 (gp91phox) was originally identified as the primary source of pathological superoxide production in insulin-resistant states, but more recently roles for Nox1 and Nox4 have been identified.46–48 A key finding in the present study is that the expression levels of Nox1 and its regulatory enzymes Noxa1/Noxo1 in the vasculature correlate with obesity-induced insulin resistance. In obese mice, the expression of Nox1, Noxa1, and Noxo1 are markedly increased and these levels are reversed by the deletion of PTP1B. Coexpression of Nox1 with Nox1 and Nox1 results in the constitutive and high-level production of superoxide.49,50 The significance of increased Nox1 activity in insulin-resistant states in not clear. The loss of Nox1 attenuates, whereas an increased expression of Nox1 potentiates, angiotensin-induced hypertension.51,52 In large conduit blood vessels, Nox1 expression is unchanged in db/db mice53; however, our study is the first to measure expression levels of these proteins in microvessels. The expression level of other Nox isoforms was examined in
addition to Nox1, and in contrast to other studies, there were no changes with obesity.

In summary, these experiments provide new evidence that in the context of obesity, the underlying risk factor that impairs both endothelium-dependent and -independent NO dilation is peripheral insulin resistance. Moderate hyperglycemia or morbid obesity does not cause endothelial dysfunction when the enzyme PTP1B is absent. These data indicate that PTP1B may represent an important therapeutic target for not only the metabolic but also cardiovascular therapy of obesity.

Acknowledgments

We gratefully acknowledge the assistance of Dr Brenda Lilly (Medical College of Georgia) in developing a PCR screening strategy for PTP1B. We also acknowledge Jessica Osmond for helpful review of the manuscript. We thank Dr David M. Stern for initial guidance and encouragement.

Sources of Funding

This work was supported by NIH grants 5R01HL075633 and 1R01HL092446 to D.J.F.; 5R01HL085827 and 1R01HL092446 to D.J.F.; AHA Pre-Doctoral Fellowship (to M.I.A.); 1R01HL092446 (to D.W.S.); 5R01HL085827 and 1R01HL092446 (to J.A.P.); and a Heart Association Established Investigator Award (to D.J.F.).

Disclosures

None.

References

29. Fendler B, Tschopp O, Hynx D, Yang ZZ, Dinholser S, Hemmings BA. Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. Mol Cell Biol. 2006;26:8042–8051.


Deletion of Protein Tyrosine Phosphatase 1b Improves Peripheral Insulin Resistance and Vascular Function in Obese, Leptin-Resistant Mice via Reduced Oxidant Tone

M. Irfan Ali, Pimonrat Ketsawatsomkron, Eric J. Belin de Chantemele, James D. Mintz, Kenjiro Muta, Christina Salet, Stephen M. Black, Michel L. Tremblay, David J. Fulton, Mario B. Marrero and David W. Stepp

Circ Res. 2009;105:1013-1022; originally published online September 24, 2009;
doi: 10.1161/CIRCRESAHA.109.206318

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/105/10/1013

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/09/24/CIRCRESAHA.109.206318.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Materials and Methods – Supplemental

**Insulin receptor phosphorylation:** An anti-IRβ antibody was used to immunoprecipitate insulin receptor proteins from 400-1000 μg of tissue lysates and phosphorylation status assess with phosphor-tyrosine antibody (PY4G10, Upstate). Samples were rocked for 2 hours at 4°C and 50 μl of protein A/G agarose beads (Santa Cruz) were added and incubated overnight at 4°C. The agarose beads were then pelleted by centrifugation at 2,000 rpm for 3 minutes. The beads were washed 3 times with ice-cold rinse buffer containing 1x phosphate buffered saline (PBS) and 1 mM of sodium vanadate. Immunoprecipitated proteins were dissolved by boiling for 5 minutes at 95°C in SDS sample buffer and were subsequently size-fractionated by SDS-PAGE and immunoblotted with anti-phosphotyrosine (PY4G10, Upstate). Membranes were stripped and re-probed with anti–IRβ antibody (C-19, Santa Cruz).

**Quantitative Assessment of Superoxide:** The abundance of super-oxide was assessed using Electron Paramagnetic Resonance (EPR) Spectroscopy. Superior mesenteric arterial cascades were dissected, pooled, and immediately immersed in PBS and 25 μM desferrioxamine. Samples were homogenized and protein concentrations normalized (2 mg/mL). Samples were then split into two volumes containing pegylated superoxide dismutase (100 U/mL) or solvent (vehicle). These mixtures were then incubated with methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH, 20μM) for 45 minutes. Samples were analyzed immediately with a MiniScope MS200 EPR.
Magnettech, Berlin, Germany) at a microwave power of 40 mW, modulation amplitude of 1,000 mG, and modulation frequency of 100 kHz. The amplitude of each EPR spectrum was analyzed using ANALYSIS software (version 2.02; Magnettech).

**Mouse genotyping:** At 7-10 days of age, mouse toes were clipped. Genomic DNA was isolated by Direct PCR lysis reagent from Viagen Biotech (Cat# 101-T). One hundred ng of genomic DNA was used for each PCR reaction using specific primers for genotyping (Table ST1). Representative genotypes of PTP1B and db/db mice are shown in Supplemental Figure S1. Deletion of PTP1B was verified further verified by Western analysis using an anti-PTP1B antibody (#07-088) from Upstate (Lake Placid, NY).

**Metabolic Phenotyping:** Growth of mice was measured via body weight twice weekly. To assess glucose tolerance, mice were fasted overnight and then anesthetized with 2% isoflurane for cannulation of the left carotid artery and jugular vein. Fasting blood glucose was assessed as the average of two initial measurements in the anesthetized mice using a Precision XL glucometer (Abbott Laboratories; Alameda, CA). An additional measurement was used to determine HbA1c levels using A1CNow from Metrika (Terrytown, NY). A 10-mg bolus of glucose was injected into each mouse, and blood glucose was measured every 5 minutes for 50 minutes.

In a second group, fasted mice were anesthetized by isoflurane in a rapid induction chamber and swiftly decapitated. Trunk blood was collected in heparin, centrifuged at 4°C to obtain plasma, and frozen for later analysis. Plasma cholesterol, free fatty acids (FFAs), and triglycerides were determined using colorimetric assays from
Wako Chemical (Richmond, VA). Plasma insulin and leptin concentrations were determined by ELISA (Alpco, Salem, NH).

**Procedure for Dihydroethidium Staining:** Microvessels of similar target size described above were excised and washed for 30 minutes in PBS. Microvessels were bathed in dihydroethidium (DHE, 200 μM, Invitrogen) for 30 minutes at 37°C. Microvessels were then removed, washed in ice-cold PBS for 1 hour, and immediately placed in Optimal Cutting Temperature Compound (Tissue-Tek, Redding, CA). The vessels were sectioned to 10 μm and mounted on Fisher premium glass slides. Microscopy was performed with a Leica DMIL inverted microscope and Leica 320 digital camera (Bannockburn, IL) at 40x magnification.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 (S1). Representative PCR gels demonstrating the genetic distinction between each mouse used in this study.

Supplemental Figure 2 (S2). Deletion of PTP1B does not affect weight gain in either control or obese mice.

Supplemental Figure 3 (S3). Glucose tolerance tests were performed on each genotype to determine the degree of insulin sensitivity in each. $K_{db}$HPTP demonstrated a marked impairment while PTP1B deletion corrected this on the $db/db$ background. Area under the curve data are reported in Table 1.

Supplemental Table 1 (ST1). Primer sequences that were used in the genotyping RT-PCR procedure and Real-Time RT-PCR are denoted in this table for reference.

Supplemental Figure 4 (S4). Protein expression of phosphorylated and total endothelial nitric oxide synthase (eNOS, normalized by GAPDH expression) was done in each mouse. Phosphorylated eNOS:total eNOS was the same across all four genotypes.

Supplemental Figure 5 (S5). Non-nitric oxide, endothelium independent vasodilation was determined across all four mice using papaverine. Dilation was similar in all four genotypes.
Supplemental Figure 6 (S6). Super-oxide scavenging restores NO-mediated vasodilation as indicated by normalized sensitivity to L-NAME.

[* = p < 0.05, (+) PEG-SOD, (+) L-NAME vs. (–) L-NAME for each mouse, n > 5]

Supplemental Table 2 (ST2). Passive mechanical data across all mice were assessed in all mice. Stress, strain and wall thickness measurements were similar in all mice. Maximum passive wall diameter and myogenic tone were unchanged as well.

Supplemental Table 3 (ST3). Numerical data for gene expression as assessed by RT-PCR (Figure 4 of the main text). [* = p < 0.05 HdbKPTP vs. HdbHPTP; † = p < 0.05 KdbHPTP vs. HdbHPTP; ‡ = p < 0.05 KdbKPTP vs. KdbHPTP; ** = p < 0.05 KdbKPTP vs. HdbHPTP]
Supplemental Figure 1 (S1)

M    1     2    3     4

[Image of gel electrophoresis]

M: DNA ladder marker
1: H<sub>db</sub>H<sub>PTP</sub>
2: H<sub>db</sub>K<sub>PTP</sub>
3: K<sub>db</sub>H<sub>PTP</sub>
4: K<sub>db</sub>K<sub>PTP</sub>

Supplemental Figure 2 (S2)

[Graph showing body weight vs. age]

Supplemental Figure 3 (S3)

[Graph showing blood glucose vs. time]
Supplemental Table 1 (ST1)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/db F</td>
<td>5' CCAACAGGTCCATACTATTAGAAGATTTTTACATTTTGATGGAG 3'</td>
</tr>
<tr>
<td>db/db R</td>
<td>5' GTCAAAACTGAACTACATCAAACCTAC 3'</td>
</tr>
<tr>
<td>NEOF</td>
<td>5' CCTGATGCTCTCTCGTCCAGATCA 3'</td>
</tr>
<tr>
<td>NEOR</td>
<td>5' TGTGCCTCGACGTTGTCACTGAAG 3'</td>
</tr>
<tr>
<td>PTP1B F</td>
<td>5' GAGATTGTGACTGCTTAAAGGCTG 3'</td>
</tr>
<tr>
<td>PTP1B R</td>
<td>5' GACTCGGGGACTCCAAAGTCAGG 3'</td>
</tr>
<tr>
<td>NOX1F</td>
<td>5’ CATGGCCTGGGTGGGATTGT 3’</td>
</tr>
<tr>
<td>NOX1R</td>
<td>5’ TGGGAGCGATAAAAGCGAAGGA 3’</td>
</tr>
<tr>
<td>NOXOIF</td>
<td>5’ ACACGTCGGGGGCATACTGGTC 3’</td>
</tr>
<tr>
<td>NOXOIR</td>
<td>5’ GGCTGCCTCTGGTGGGATA 3’</td>
</tr>
<tr>
<td>NOXA1F</td>
<td>5’ ACGGTGGATGTTCCTGTGTA 3’</td>
</tr>
<tr>
<td>NOXA1R</td>
<td>5’ AAGCATGGCTTCCACATAGG 3’</td>
</tr>
<tr>
<td>NOX2F</td>
<td>5’ CAAGATGGAGGTGGGACAGT 3’</td>
</tr>
<tr>
<td>NOX2R</td>
<td>5’ GCTTATCACAGCCACACAGCA 3’</td>
</tr>
<tr>
<td>NOX4F</td>
<td>5’ TGTTCATGTGTTTCAGGGTG 3’</td>
</tr>
<tr>
<td>NOX4R</td>
<td>5’ AAAACCCTCGAGGCAAAGAT 3’</td>
</tr>
<tr>
<td>SOD1F</td>
<td>5’ CCAGTCAGGGACCTCATT 3’</td>
</tr>
<tr>
<td>SOD1R</td>
<td>5’ TTGTGGTCATGGACACCACA 3’</td>
</tr>
<tr>
<td>SOD2F</td>
<td>5’ CCGTCCTGCCCCCTCGCTGATG 3’</td>
</tr>
<tr>
<td>SOD2R</td>
<td>5’ GCACGCGCCCGGACAAA 3’</td>
</tr>
<tr>
<td>SOD3F</td>
<td>5’ ATCCCACAAGGCCCTAGTCT 3’</td>
</tr>
<tr>
<td>SOD3R</td>
<td>5’ GTGCTATGGGACAGGAAGA 3’</td>
</tr>
<tr>
<td>GAPDHF</td>
<td>5’ ACCCAGAAGACTGTGGATG 3’</td>
</tr>
<tr>
<td>GAPDHR</td>
<td>5’ CACATTGGGGAAGAACAC 3’</td>
</tr>
</tbody>
</table>
Supplemental Table 2 (ST2)

<table>
<thead>
<tr>
<th>Passive Mechanical Properties of vessels in all genotypes</th>
<th>H_{db}H_{PTP}</th>
<th>H_{db}K_{PTP}</th>
<th>K_{db}H_{PTP}</th>
<th>K_{db}K_{PTP}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall Thickness at 120 mmHg (μm)</td>
<td>16±1.0</td>
<td>14±1.3</td>
<td>17±1.0</td>
<td>16±1.8</td>
</tr>
<tr>
<td>Wall:Lumen Ratio at 120 mmHg</td>
<td>.34±.04</td>
<td>.33±.05</td>
<td>.35±.03</td>
<td>.33±.04</td>
</tr>
<tr>
<td>Circumferential Stress at 120 mmHg (N/m²)</td>
<td>.46±.05</td>
<td>.55±.07</td>
<td>.36±.03</td>
<td>.56±.11</td>
</tr>
<tr>
<td>Circumferential Strain at 120 mmHg</td>
<td>.68±.06</td>
<td>.80±.05</td>
<td>.66±.10</td>
<td>.73±.14</td>
</tr>
<tr>
<td>β-coefficient</td>
<td>3.3±.3</td>
<td>2.9±.1</td>
<td>3.2±.4</td>
<td>3.3±.5</td>
</tr>
<tr>
<td>Average Maximum Passive Wall Diameter (μm)</td>
<td>110±6</td>
<td>94±4</td>
<td>109±5</td>
<td>115±8</td>
</tr>
<tr>
<td>Average Myogenic Tone (% from baseline, non-stimulated active diameter)</td>
<td>26±2</td>
<td>28±2</td>
<td>30±3</td>
<td>28±2</td>
</tr>
<tr>
<td>Gene Expression (2^{-ΔΔCt})</td>
<td>H_{db}H_{PTP}</td>
<td>H_{db}K_{PTP}</td>
<td>K_{db}H_{PTP}</td>
<td>K_{db}K_{PTP}</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>NOX1</td>
<td>1.45 ± .23</td>
<td>0.48 ± .21</td>
<td>3.52 ± 1.6†</td>
<td>1.31 ± .41‡</td>
</tr>
<tr>
<td>NOXA1</td>
<td>1.17 ± .11</td>
<td>0.05 ± .01*</td>
<td>9.94 ± 1.5†</td>
<td>0.52 ± .10‡</td>
</tr>
<tr>
<td>NOXO1</td>
<td>1.14 ± .14</td>
<td>0.83 ± .17</td>
<td>8.81 ± 2.78†</td>
<td>1.67 ± .55‡</td>
</tr>
<tr>
<td>NOX2</td>
<td>1.28 ± .09</td>
<td>0.66 ± .17</td>
<td>0.97 ± .10</td>
<td>1.47 ± .18</td>
</tr>
<tr>
<td>NOX4</td>
<td>1.14 ± .13</td>
<td>2.56 ± .65</td>
<td>2.74 ± 1.4</td>
<td>2.47 ± .87</td>
</tr>
<tr>
<td>SOD1</td>
<td>1.24 ± .22</td>
<td>2.04 ± .40</td>
<td>1.03 ± .31</td>
<td>1.66 ± .37</td>
</tr>
<tr>
<td>SOD2</td>
<td>1.15 ± .23</td>
<td>1.53 ± .34</td>
<td>1.00 ± .50</td>
<td>3.36 ± .52**†</td>
</tr>
<tr>
<td>SOD3</td>
<td>1.24 ± .12</td>
<td>3.07 ± .88*</td>
<td>0.68 ± .10</td>
<td>4.76 ± 1.6**‡</td>
</tr>
</tbody>
</table>