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## COMMENTARY

# Protein Tyrosine Phosphatase-1B in Diabetes

*Brian P. Kennedy* and Chidambaram Ramachandran*

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, MERCK FROSST CENTER FOR THERAPEUTIC RESEARCH, POINTE CLAIRE-DORVAL, QUEBEC, CANADA H9R 4P8

**ABSTRACT.** A role for protein tyrosine phosphatases in the negative regulation of insulin signaling and a putative involvement in the insulin resistance associated with type 2 diabetes have been postulated since their discovery. The recent demonstration that mice lacking the protein tyrosine phosphatase-1B (PTP-1B) have enhanced insulin sensitivity validates this. Furthermore, when fed a high fat diet, these mice maintained insulin sensitivity and were resistant to obesity, suggesting that inhibition of PTP-1B activity could be a novel way of treating type 2 diabetes and obesity. This commentary reviews our current knowledge of PTP-1B in insulin signaling and its role in diabetes and discusses the development of potent and selective PTP-1B inhibitors. BIOCHEM PHARMACOL **60**;7:877–883, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** PTP-1B; diabetes; insulin signaling; obesity

Phosphorylation of protein tyrosyl residues is a controlling event that either activates or attenuates intracellular signaling pathways involved in cell proliferation, differentiation, and metabolism. The equilibrium established by the enzymes that control the level of tyrosine phosphorylation, the protein tyrosine kinases and the PTPs,† dictates the degree of signaling within the particular pathway. The IR is a tyrosine kinase composed of two extracellular ligand-binding α subunits linked by disulfide bonds to two transmembrane β subunits containing tyrosine kinase domains [1, 2]. Insulin initiates its biological effects by binding to the α subunits, thereby activating the intrinsic tyrosine kinase activity of the β subunits. This results in autophosphorylation of critical tyrosine residues in the regulatory domain to fully activate the tyrosine kinase activity of the IR, which then is capable of phosphorylating its various substrates to propagate the insulin signal [2, 3]. Within the past 10 years since the identification of the PTPs, it has been speculated that some specific PTP(s) is involved in the dephosphorylation, and thus inactivation of the IR and attenuation of the insulin signal [4, 5]. It has also been hypothesized that disequilibrium in enzyme activity between the IR and this PTP could be a contributing factor to the insulin resistance observed in DM2 or NIDDM [5, 6]. Inhibition of the PTP specific for the IR would be beneficial in the treatment of this disease, since it would result in the maintenance of an activated IR and hence prolong the insulin signal. Consistent with this proposal, vanadate, which is a non-specific PTP inhibitor, can improve blood glucose levels in DM2 [7, 8].

Recently, we have shown, using a gene targeting approach, that mice lacking PTP-1B have enhanced insulin sensitivity [9]. Treatment of these mice with insulin results in an increased and prolonged tyrosine phosphorylation of the IR in liver and muscle. These results suggest that PTP-1B may be the IR-specific PTP and would be a good therapeutic target in the treatment of DM2. In consideration of this exciting new finding, this commentary will focus on reviewing the role of PTP-1B in diabetes and the progress towards the identification of potent, selective PTP-1B inhibitors. For additional recent reviews on PTP activity in insulin and intracellular signaling in general, see Refs. 10–14.

## IS PTP-1B INSULIN RECEPTOR SPECIFIC?

PTPs display very little substrate specificity when assayed *in vitro* either in the test tube or by overexpression in cell lines. This has led to the erroneous concept that PTPs are promiscuous and have multiple unrelated cellular substrates. Only recently, with the generation of mice having targeted mutations in various PTP genes, has the *in vivo* specificity of these enzymes been demonstrated. This is illustrated by the unique phenotypes in mice in which a particular PTP gene has been mutated. For example, the loss of CD45 leads to a defect in the development of T cells as well as a defect in the signaling by T and B cells [15]. Ablation of SHP-2 [16], PTP-PEST [17], PTP-σ [18], or TC-PTP [19] is lethal at various stages of development, and cells derived from these embryos all have unique phenotypes. Loss of SHP-1, as in *motheaten* mice, leads to

* Corresponding author: Dr. Brian P. Kennedy, Department of Biochemistry and Molecular Biology, Merck Frosst Center for Therapeutic Research, Merck Frosst Canada Inc., P.O. Box 1005, Pointe Claire-Dorval, Quebec, Canada H9R 4P8. Tel. (514) 428-8548; FAX (514) 428-8615; E-mail: brian_kennedy@merck.com

† *Abbreviations*: DM2, diabetes mellitus type 2; EGF(R), epidermal growth factor (receptor); $F_2$PMP; difluoromethylene phosphonophenylalanine; IR, insulin receptor; LAR, leukocyte antigen-related tyrosine phosphatase; NIDDM, non-insulin-dependent diabetes mellitus; PTP, protein tyrosine phosphatase; and QTL, quantitative trait locus.

enhanced activation of certain immune response signals [20]. Loss of LAR appears to cause a defect in lactation [21], although there are some conflicting mouse data on its involvement in insulin signaling [22, 23]. The loss of PTP-1B, as has been pointed out earlier, causes an enhanced insulin sensitivity and resistance to diet-induced obesity [9]. In all these genetic studies, other PTPs that were expressed in the various target tissues were not able to compensate for the loss of function of the deleted PTP, leading to the selective phenotype. Based on this limited number of mouse targeted mutations, these observations would suggest that most PTPs serve a unique, non-redundant function.

Initially, because PTPs were considered to be non-selective in their substrate preference, one concern was that the loss of PTP-1B activity would result in tumor formation due to the inappropriate or prolonged activation of multiple growth factor receptors. However, PTP-1B$^{-/-}$ mice were normal in size compared with their wild-type littermates and were long-lived without any significant incidence of tumor formation in old age compared with controls [Tremblay ML and Kennedy BP, unpublished results]. For example, the activated EGFR was suggested to be a substrate for PTP-1B [24, 25]. Examination of tyrosine phosphorylation levels of EGFR in primary hepatocytes prepared from PTP-1B$^{-/-}$ mice and wild-type littermates treated with EGF revealed that there was no difference in the levels of EGFR tyrosine phosphorylation between the two samples. In addition, tyrosine phosphorylation levels of the IGF-1 receptor, which is structurally highly homologous to IR, were unchanged between PTP-1B-deficient and wild-type mice. In PTP-1B-deficient mice, only the metabolic insulin signaling pathway appeared to be affected; no overt mitogenic effects were observed.

Where in the insulin signaling pathway does PTP-1B act? Insulin treatment of PTP-1B$^{-/-}$ mice results in increased tyrosine phosphorylation of the IR. There are two possibilities that could explain how the loss of PTP-1B activity could achieve this: (i) PTP-1B directly interacts and dephosphorylates the activated receptor; and (ii) PTP-1B acts indirectly by activation of some other signaling protein, perhaps another PTP, that is responsible for the direct dephosphorylation of the IR. There is evidence in support of a direct interaction with the IR. Catalytically inactive PTP-1B mutants that have had the active site cysteine replaced by serine have been shown to coprecipitate with the fully phosphorylated (P-Tyr$_{1146}$, P-Tyr$_{1150,1151}$) activated IR [26, 27]. It is also of interest to note that PTP-1B displays selectivity compared with CD45, LAR, and TC-PTP in dephosphorylation of a triphosphotyrosyl IR regulatory domain peptide by preferentially dephosphorylating the two adjacent phosphotyrosines first [28]. In addition, several inhibitors that contain two adjacent nonhydrolysable $F_2$PMP phosphotyrosine analogues are potent inhibitors of PTP-1B [29]. However, if PTP-1B interacts directly with the activated IR, one of the issues to be addressed is how PTP-1B gets to its substrate. The IR is located in the plasma membrane, whereas PTP-1B localizes to the endoplasmic reticulum with its phosphatase domain directed towards the cytoplasm [30]. A possible explanation would be that some PTP-1B is associated with the plasma membrane, bringing it into close proximity with the IR. It is also known that once insulin binds to the receptor, the activated receptor is internalized quickly into endosomes, from which the receptors can be recycled or degraded by delivery to secondary lysosomes [31]. Recycled receptors are dephosphorylated prior to their return to the plasma membrane [31]. It is possible that during internalization and its transit inside the cell the IR is brought into contact with PTP-1B. The mechanism whereby PTP-1B interacts with the IR, if there is a direct interaction, and where it is positioned within the insulin signaling pathway will require further investigation.

## PTP-1B IN DIABETES

DM2 is characterized by a resistance of insulin-sensitive tissues, such as muscle, liver, and fat, to insulin action [32–36]. Although the mechanism of the insulin resistance is unknown, it is tightly associated with obesity. Approximately three-quarters of obese individuals will develop DM2. PTP-1B is involved in dephosphorylation of the activated IR and consequently in termination of the insulin signal. Therefore, any changes in expression levels or activity of PTP-1B relative to the IR could affect insulin signaling and possibly contribute to the insulin resistance observed in DM2. There are a number of examples in which increased expression of PTP-1B decreases the amount of IR tyrosine phosphorylation [11]. These observations have been made in various cell lines in which PTP-1B was overexpressed, although the caveat with these types of experiments is that other PTPs have also been shown to duplicate this result [10]. In animal studies, mice deficient in the G-protein subunit $G_{i\alpha 2}$ have a phenotype of insulin resistance that resembles DM2, which correlates with increased levels of PTP-1B expression and PTP activity in adipose tissue, liver, and muscle [37]. In animal models of DM2, such as *ob/ob* and *db/db* mice and *fa/fa* Zucker rats, expression levels of PTP-1B and other PTPs as well as PTP activity have been measured, but with conflicting results. In liver cytosol and particulate fractions of *ob/ob* mice, PTP activity, measured by dephosphorylation of the $^{32}$P-labeled insulin receptor peptide 1142–1153, resulted in a decrease in PTP activity in one study [38], in contrast to another study where an increase in activity was observed [39]. Similar conflicting results have been reported for skeletal muscle PTP activity in Zucker (*fa/fa*) rats [40, 41]. This has also been the case for diabetes chemically induced in animals using streptozotocin [42–44], alloxan [45], and goldthioglucose [46]. The reason for these discrepancies is probably the variability in the PTP assays used, as well as the multifactorial complexity of these diabetic models.

In human studies, again contradictory results have been published. PTP activity and also PTP-1B and other PTP

protein levels have been measured in muscle and adipose tissue biopsies from both insulin-resistant obese nondiabetic and obese DM2 subjects. In the particulate fraction from muscle of insulin-resistant obese non-diabetic individuals, two reports have shown that both PTP activity and PTP-1B protein levels are increased [5, 47]. The opposite findings, decreased PTP activity and PTP-1B levels, have also been reported [48]. The results from obese DM2 individuals seem to be a bit more consistent in that they all show a decrease in PTP activity either in soluble or particulate muscle fractions and a decreased PTP-1B protein expression [47–49]. In adipose tissue from obese non-diabetic subjects, protein levels of PTP-1B and the PTPs LAR and SH-PTP2 have been found to be elevated [50]. However, there is some confusion over the degree to which PTPs, particularly PTP-1B and LAR, contribute to the PTP activity observed. Ahmad *et al.* [50] report that adipose lysates from obese individuals have increased PTP activity as measured by dephosphorylation of $^{32}$P-labeled IR compared with lean controls. The increased PTP activity could be lowered to control values by immunodepletion of lysates with an anti-LAR antibody, but not with an anti-PTP-1B or SH-PTP2 antibody. Conversely, Cheung *et al.* [51], using immunodepletion of PTP-1B and a $^{32}$P-labeled peptide corresponding to the triphosphotyrosyl regulatory domain of IR to measure PTP activity, recently reported that PTP-1B constituted approximately 75% of PTP activity in adipose lysates. In this study, even though there were increased levels of PTP-1B protein, the specific activity of the enzyme apparently was decreased in obese non-diabetic and obese DM2 adipose lysates compared with control [51]. The reason for this supposed loss of PTP-1B activity is unknown.

Presently, it is very difficult, based on the above published data, to make any definitive conclusions on the involvement of PTP-1B in human DM2 or insulin resistance. The way PTP activity was measured and the small number of subjects used probably contribute to the inconsistency observed in these studies. In addition, although PTP protein levels in insulin-resistant muscle and adipose tissues have been determined, the amount of expression relative to some control protein is absent in all the studies. Therefore, it is hard to rule out the possibility that changes in PTP expression levels simply reflect a general change in abundance of all cellular proteins due to the diseased state of the tissue. Perhaps what is required to clarify these issues is to reexamine the role of PTP-1B in diabetes in light of what is known from the PTP-1B$^{-/-}$ mice study. PTP-1B$^{-/-}$ and heterozygous mice display enhanced insulin sensitivity, suggesting that loss or decrease in PTP-1B enzyme activity would promote insulin sensitivity. Therefore, the apparent increase in PTP-1B levels during obesity could potentially contribute to the insulin resistance. However, this is only part of the story, as the levels of IR also decrease during insulin resistance [52, 53]. If the activated IR is the primary substrate for PTP-1B, one really should be examining the changes in the ratio of IR and PTP-1B expression levels. For example, if PTP-1B levels remained constant in the insulin-resistant state, whereas IR levels decreased, this would have a negative effect on the steady-state level of IR tyrosine phosphorylation. If, on the other hand, there are increased levels of PTP-1B in insulin resistance, as suggested by the above studies, then there would be an even more profound negative effect on IR tyrosine phosphorylation. In fact, we have measured PTP-1B and IR levels in muscle from lean *ob*/+ controls and *ob*/*ob* mice and found significant changes in this ratio [Larsson-Forsell PKA and Kennedy BP, unpublished results]. In *ob*/*ob* mice, the levels of both PTP-1B and IR protein decrease. However, there is a much greater reduction in IR, such that in the muscle of *ob*/*ob* mice there is nearly a 6-fold increase in the ratio of PTP-1B to IR compared with *ob*/+ mice. Furthermore, it will also be important to determine how much of the PTP activity present in these tissue extracts is due to PTP-1B. With the development of selective PTP-1B inhibitors (see below), this may now be possible. Using more carefully controlled studies, with larger subject populations and the new selective PTP-1B inhibitors, it should be possible to investigate the course of PTP-1B expression and activity during the development of insulin resistance and eventually DM2.

Genetically linking PTP-1B to diabetes and obesity would certainly validate it as a factor in these disorders. Recently, the mapping of a major human QTL to chromosome 20q13.1-q13.2 that is associated with obesity and hyperinsulinaemia has been reported [54, 55]. QTL mapping uses a set of genetic markers in a genome scan to determine if any of the markers can be associated statistically with some particular phenotype. The human PTP-1B gene also maps to the identical chromosomal location, 20q13.1-q13.2 [56], making it a possible candidate gene for this obesity QTL. Since the loss of PTP-1B activity contributes to a leanness phenotype, then for some genetic change in the PTP-1B gene to be associated with obesity it would have to cause either increased PTP-1B gene expression or enzyme activity. It is also unknown whether increasing PTP-1B levels or activity could promote obesity, although in some studies (see above) increased PTP-1B levels appear to correlate with obesity. A number of other possible candidate genes are also located within this chromosomal region, so it will be interesting to see which of these genes are responsible for this QTL.

## OBESITY-RESISTANT PHENOTYPE OF PTP-1B$^{-/-}$ MICE

One of the more surprising phenotypes of the PTP-1B-deficient mice is their resistance to diet-induced obesity. Insulin is a powerful anabolic hormone that stimulates the storage of carbohydrates and fat. If PTP-1B$^{-/-}$ mice are so insulin-sensitive, why are they resistant to storing fat? One possible explanation is that PTP-1B$^{-/-}$ mice display tissue-specific insulin sensitivity. Muscle and liver appear to have increased insulin sensitivity, whereas adipose tissue is un-

changed or has a slightly reduced response to insulin compared with wild-type mice, as determined by insulin-stimulated IR tyrosine phosphorylation [9]. This would imply that adipose tissue in PTP-1B$^{-/-}$ mice might have reduced lipogenic activity. The reason for this is not clear but suggests that PTP-1B may have slightly different functions in fat than in muscle and liver. Another possibility is that mice lacking PTP-1B have increased insulin sensitivity in the brain. Injection of insulin into the brains of rats results in decreased feeding [57–59]. It has been postulated that one of the purposes for the hyperinsulinaemia that accompanies weight gain is to increase brain insulin levels in order to reduce food intake [57]. Interestingly, low concentrations of vanadate, when microinjected into the lateral cerebral ventricle of rats, strongly suppress food intake and weight gain [60]. However, because of the tissue-specific insulin sensitivity observed in these animals, it needs to be established whether or not PTP-1B$^{-/-}$ mice have increased insulin sensitivity in the brain. Obviously, these areas require further investigation to determine how they contribute to the obesity resistance observed in the PTP-1B-deficient mice.

## PTP-1B INHIBITORS

The development of selective PTP-1B inhibitors would certainly help in clarifying the role of PTP-1B in diabetes. This will be a daunting challenge due to the large number of PTP family members and the conservation of the PTP catalytic domain [13, 61]. Progress in developing selective PTP inhibitors is being made, although most inhibitors described to date are non-selective. One of the best characterized is vanadate, which is a reversible non-selective inhibitor of PTP with affinity in the micromolar range [62]. This is presumably due to the ability of vanadate to adopt a trigonal bipyramidal structure mimicking the transition state of phosphoryl transfer reactions [62]. In the structure of *Yersinia* PTP with vanadate, the vanadium atom is at a distance of 2.5 Å from the active site thiol [63]. The inhibition of an insulin receptor-specific PTP by vanadate is thought to be the major reason for the insulin mimetic effect of this compound. Although vanadate is a non-selective inhibitor of PTP, its ability to inhibit PTP in intact cells depends on the redox state of the cell [64]. This may account for its ability to normalize blood glucose in animal models of diabetes with minimal toxicity at doses that are efficacious. Several analogues of vanadate have been synthesized, most of which either increase the permeability or alter the complexation properties of vanadate [65, 66]. Vanadate has been used in a number of clinical trials and was shown to be effective in improving insulin sensitivity in DM2 subjects [7, 8].

PTPs contain a catalytic cysteine residue, and not surprisingly, alkylating and oxidizing agents are potent inhibitors of PTP. Peroxovanadate, which is a potent inhibitor of PTPs, causes irreversible oxidation of the active site cysteine to sulphonic acid [62]. It shows specificity towards PTPs compared with other active site cysteine-containing enzymes, resulting in a strong enhancement of protein tyrosine phosphorylation in treated cells. A series of peroxovanadium complexes with several ancillary ligands have been synthesized, some of which have been shown to increase the level of insulin receptor phosphorylation [67].

PTP substrate analogues in which the cleavable O-P linkage of phosphotyrosine has been replaced by PTP-resistant O-S (sulfate), O-P-S (thiophosphate), C-P (methylene phosphonate), and O-C (malonyl) turn out to be potent inhibitors of PTPs [68–74]. Their affinities are approximately the same as those of the parent phosphotyrosine-containing peptides. It has been shown that peptide sequences around phosphotyrosine play a critical role in modulating the affinity of the peptide towards PTP [75]. In addition, slight changes in the structure of the phosphotyrosine mimetic also can affect potency drastically. Replacement of the methylene hydrogens with fluorine in the phosphotyrosine analogue methylene phosphonophenylalanine resulted in only a modest change in potency with respect to SH2 domain binding, whereas the same modification in a PTP-1B inhibitory peptide enhanced the potency of the inhibitor peptide by about 1000-fold [71]. The enhancement in potency by fluorine substitution was shown not to be a p$K_a$ effect, but rather to be due to a more favorable interaction with the enzyme [72]. Using $F_2$PMP as a potent active site target residue, we have developed a novel technique to investigate the inhibitor binding specificity of PTP-1B [76]. Starting with the EGFR autophosphorylation site sequence [DADE(pY)L], the $F_2$PMP hexapeptide was synthesized and shown to be a potent ($K_i$ = 26 nM) PTP-1B inhibitor. A library of peptides in which each amino acid in the hexapeptide (except $F_2$PMP) was replaced systematically with 18 naturally occurring amino acids was then prepared, and an affinity selection process was used to identify the best tight-binding inhibitors. Using this approach, it was demonstrated that acidic residues N-terminal and aromatic residues C-terminal to $F_2$PMP greatly enhanced PTP-1B binding, and the most potent inhibitor identified in the library displayed this property, EEDE($F_2$PMP)M, $K_i$ = 7.2 nM.

In a similar fashion, except starting with a tripeptide library containing $F_2$PMP and testing members individually for their ability to inhibit a panel of PTPs, we have been able to identify potent and selective PTP-1B inhibitors [29]. The $IC_{50}$ for the most potent PTP-1B inhibitor identified, Glu-$F_2$PMP-$F_2$PMP ($IC_{50}$ = 40 nM), was at least 100-fold lower than the $IC_{50}$ values obtained for CD45 ($IC_{50}$ = 7,500 nM), PTP β ($IC_{50}$ = 4,200 nM), LAR ($IC_{50}$ = 45,000 nM), and SHP-1 ($IC_{50}$ = 6,000 nM). However, replacement of the N-terminal glutamic acid with proline in the above peptide decreases its potency to inhibit PTP-1B ($IC_{50}$ = 300 nM) but increases its potency to inhibit PTP β ($IC_{50}$ = 200 nM). These studies demonstrate that it is possible to identify potent and selective inhibitors for PTPs, and replacement of a single amino acid can alter

not only the potency but also the selectivity of these inhibitors.

Although these peptide inhibitors are the most potent and selective PTP-1B inhibitors identified, the fact that they are peptide phosphonates makes them less desirable as drug candidates. There is a need for non-peptidic PTP inhibitors. It has been observed that simple aryl phosphates such as *p*-nitrophenyl phosphate are hydrolysed quite efficiently by tyrosine phosphatase [77]. Based on this observation, aryl phosphonates have been made and tested as inhibitors of PTPs [78–83]; whereas phenyl difluoromethylenephosphonate was a poor inhibitor, naphthalene difluoromethylenephosphonate was quite potent and inhibited PTP-1B with an $IC_{50}$ of 40–50 μM. Various other aryl phosphonate inhibitors have been made, and it has been found that inhibitors that contain two phosphonate moieties are significantly more potent than those containing one. Some of these inhibitors have low micromolar $IC_{50}$ values, and it is believed that the added potency is due to binding of the second phosphonate to a secondary non-catalytic phosphotyrosine binding site in PTP-1B [84]. In addition, these inhibitors show some selectivity towards PTP-1B when tested on other PTPs. Recently, a number of non-peptidic non-phosphonate PTP-1B inhibitors were described [85] that showed selectivity over several other PTPs that were tested. More importantly, some of the compounds were reported to have a significant effect on reducing plasma glucose and insulin levels in *ob/ob* mice. These observations were the first reported pharmacological proof that PTP-1B inhibitors have antidiabetic activity. The fact that one can develop potent selective PTP-1B inhibitors, some of which are starting to show efficacy in diabetic animal models, bodes well for the eventual development of compounds that can be tested in humans.

## CONCLUSION

PTP-1B-deficient mice are resistant to both diabetes and obesity. Drugs that inhibit PTP-1B activity have the potential to be important new therapies in the treatment of these prevalent metabolic disorders. Much more work will be required to better understand the role of PTP-1B in insulin signaling and its potential involvement in insulin resistance in DM2. Furthermore, the development of selective, potent, and bioavailable inhibitors of PTP-1B will be a formidable challenge, although some of the groundwork has now been laid out. The possibility of a compound beneficially affecting both diabetes and obesity would have tremendous potential.

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