

Diabetes Reduces Autophosphorylation of Retinal Insulin Receptor and Increases Protein-Tyrosine Phosphatase-1B Activity

Raju V. S. Rajala,^{1,2,3} Brandt Wiskur,⁴ Masaki Tanito,^{1,3,5} Michelle Callegan,^{1,3,6} and Ammaji Rajala^{1,3}

PURPOSE. Protein-tyrosine phosphatase-1B (PTP1B) has been implicated in the negative regulation of insulin signaling. The expression, activity, and functional role of PTP1B in the retina are unknown. In this study, the authors examined the relationship between the retinal insulin receptor (IR) and PTP1B in normal and diabetic mouse retinas.

METHODS. IR and PTP1B localization was examined by immunohistochemistry. The activation of IR was analyzed using specific antibodies against phosphotyrosine. PTP1B activity was determined in anti-PTP1B immunoprecipitates. Glutathione-S-transferase fusion proteins containing wild-type and catalytically inactive mutant PTP1B was used to study the interaction between IR and PTP1B. Anti-IR immunoprecipitates and the cytoplasmic domain of purified IR were incubated in the presence of ATP, and the autophosphorylation of IR with antiphosphotyrosine antibody was analyzed.

RESULTS. Immunohistochemical analysis of PTP1B shows that it is predominantly expressed in nonphotoreceptor layers of the retina, though it is clearly expressed in the inner segments of the rod photoreceptors. The IR is predominately expressed in rod inner segments. Biochemical analysis of rod outer segments indicates the presence of IR and PTP1B. Retinal IR exhibits a high level of basal autophosphorylation, and this autophosphorylation is reduced in diabetic mouse retinas. In vitro, PTP1B is able to dephosphorylate the autophosphorylated IR. Substrate mutant-trap results indicate a stable interaction between IR and PTP1B. Further, PTP1B activity was increased in diabetic mouse retinas.

CONCLUSIONS. These studies indicate that diabetes reduces the autophosphorylation of retinal IR and increased PTP1B activity. Further, PTP1B regulates the state of IR phosphorylation in the retina. (*Invest Ophthalmol Vis Sci.* 2009;50:1033-1040) DOI: 10.1167/iovs.08-2851

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Insulin receptors (IRs) and insulin signaling proteins are widely distributed throughout the central nervous system (CNS). Dysregulation of insulin signaling in the CNS has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer and Parkinson diseases.^{1,2} Cells of bovine and rat retina contain high affinity receptors for insulin.³ Retinal IR is constitutively active; however, this constitutive activation is impaired in diabetic retinopathy.⁴ Further, IR signaling provides a trophic signal for transformed retinal neurons in culture,⁵ and we recently reported that deletion of IRs from rod photoreceptors resulted in stress-induced photoreceptor degeneration.⁶

The extent of tyrosyl phosphorylation on a given protein is controlled by the reciprocal action of protein-tyrosine kinase and protein-tyrosine phosphatase (PTP) activities. Specific PTPs, including LAR, SHP-2, and protein-tyrosine phosphatase-1B (PTP1B), have been implicated in the regulation of normal IR signaling.⁷⁻²⁰ Of these, PTP1B has received significant attention because it is an abundant enzyme expressed in all insulin-sensitive tissues.^{21,22} PTP1B is an abundant, widely expressed nonreceptor tyrosine phosphatase thought to be a key negative regulator of insulin signaling.^{23,24} It has previously been shown that PTP1B overexpression results in the inhibition of IR and IRS-1^{14,19,25}; furthermore, introduction of anti-PTP1B antibodies into cells enhances IR signaling.²⁶ Global deletion of PTP1B in mice results in increased systemic insulin sensitivity, enhanced glucose uptake into skeletal muscle, and improved glucose tolerance.^{27,28} Increased and prolonged tyrosine phosphorylation of the IR was also observed in mice lacking PTP1B.^{27,28} The increased insulin sensitivity is attributed to the absence of PTP1B and results from failure to dephosphorylate the IR.^{27,28}

In this study we observed increased basal retinal IR autophosphorylation compared with liver samples. In diabetes, the IR autophosphorylation was reduced, and we hypothesized that this decreased IR autophosphorylation could be the result of increased PTP1B activity. Consistent with our hypothesis, we observed significantly increased PTP1B activity. In this study we also demonstrated a stable interaction between the IR and PTP1B. Furthermore, our studies demonstrate that PTP1B regulates the state of IR phosphorylation in the retina.

MATERIALS AND METHODS

Materials

Polyclonal anti-PTP1B, PTP1B substrate RRLIEDAE_YYAARG, and phosphatase assay reagents were obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal PY-99 and polyclonal anti-IR β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific polyclonal anti-IR/IGF-1R (pYpYp^{Y1158/1162/1163}) antibody was obtained from Biosource International (Camarillo, CA). The actin antibody was obtained from Affinity BioReagents (Golden, CO). A quick-change, site-directed mutagenesis kit was obtained from Strat-

agene (La Jolla, CA). All other reagents were of analytical grade and from Sigma.

Animals

All animal work was conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. Mice were born and raised in our vivarium and kept under dim cyclic light (5 lux, 12 hours on/12 hours off, 7 am-7 pm) before experimentation. In all experiments, rats and mice were humanely killed by asphyxiation with carbon dioxide before the retinas were harvested.

Generation of Hyperglycemic Mice

Hyperglycemia was induced by a series of two injections. At 8 and 9 weeks, C57BL6/J mice were weighed and given intraperitoneal injections (100 mg/kg) of streptozotocin (STZ) in freshly dissolved citrate buffer (10 mmol, pH 4.5). Control animals were given intraperitoneal injections of citrate buffer only. At 10 weeks, mice were weighed, and blood glucose levels were analyzed. The average weight was 16.33 ± 0.77 g for diabetic mice and 17.63 ± 0.55 g for nondiabetic mice ($P = 0.11$). The average blood glucose level was 433.75 ± 36.59 mg/dL for diabetic mice and 132.25 ± 14.57 mg/dL for nondiabetic mice ($P = 0.005$). Mice with blood glucose levels greater than 250 mg/dL (TrueTrack Smart System; AR-MED Ltd., Egham, UK) were considered hyperglycemic. Retinas were immediately removed after euthanasia and were frozen in liquid nitrogen.

Cloning, Expression, and Purification of PTP1B

Retinal PTP1B was obtained by PCR of reverse-transcribed mouse retinal RNA using 5' and 3' oligonucleotides designed based on mouse PTP1B²⁹ (accession number NP_035331; sense, GAA TTC ATG GAG ATG GAG AAG GAG TTC GAG; antisense, GTC GAC TCA GTG AAA ACA CAC CCG GTA GC). Site-directed mutagenesis was carried out on PTP1B to substitute aspartic acid 181 to alanine (D181A) according to the method described previously.³⁰ Primers used in site-directed mutagenesis are as follows: (sense, ACC ACA TGG CCT GCC TTT GGA GTC CCC; antisense, GGG GAC TCC AAA GGC AGG CCA TGT GGT). PCR products were cloned into TOPO sequencing vector (Invitrogen), and the sequences were verified by DNA sequencing. Wild-type and mutant cDNAs were sequenced after PCR and were excised from the sequencing vector as *EcoRI/SalI* and cloned into the GST fusion vector pGEX-4T1.

Expression of GST-Fusion Proteins

Overnight culture of *Escherichia coli* BL21 (DE3; pGEX-PTP1B and pGEX-PTP1B-D181A) was diluted 1:10 with LB containing 100 µg/mL ampicillin grown for 1 hour at 37°C and induced for another hour by the addition of isopropyl β-D-1-thiogalactopyranoside to 1 mM. Bacteria were sonicated three times for 20 seconds each time in lysis buffer containing 10 mM imidazole-HCl (pH 7.2), 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, and 1% Triton X-100. Lysates were clarified by centrifugation, and the supernatants were incubated with 500 µL of 50% glutathione-coupled beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 30 minutes at 4°C. GST-PTP1B fusion proteins were washed in lysis buffer and eluted twice with 1 mL of 5 mM reduced glutathione (Sigma) in phosphatase buffer (20 mM Tris [pH 7.4], 5% glycerol, 0.05% Triton X-100, 2.5 mM MgCl₂, aprotinin [2 µg/mL], and leupeptin [5 µg/mL]). Glycerol was added to a final concentration of 33% (vol/vol), and aliquots of enzyme were stored at -20°C.

Immunohistochemistry

Preparation of retinal tissue sections for immunohistochemistry were described previously.³¹ Eucleated eyes were immersed in 4% parafor-

maldehyde containing 20% isopropanol, 2% trichloroacetic acid, and 2% zinc chloride for 24 hours at room temperature. The eyes were embedded in paraffin, and 4-µm-thick sections containing the whole retina, including the optic nerve head, were cut along the vertical meridian of the eyeball. Endogenous peroxidase activity was inactivated with 3% H₂O₂ for 10 minutes, and the antigen was retrieved by heating in a microwave oven in 10 mM citrate buffer for 8 minutes. Sections were blocked with serum-free blocking reagent (Dako, Carpinteria, CA) for 1 hour at room temperature and incubated overnight at 4°C with the anti-PTP1B or IR antibody diluted with antibody diluent (Dako) and then with the peroxidase-linked anti-rabbit IgG polymer (EnVision+ System; Dako) for 1 hour at 37°C. Signals were developed with 3',3'-diaminobenzidine (Dako) as chromogen and were visualized with a light microscope (Eclipse E800; Nikon, Tokyo, Japan).

PTP1B Activity Assay

In vitro PTP activity assay was conducted based on a protocol previously described.³² Retinas were lysed in solubilization buffer (phosphate-buffered saline) containing 1% Igepal, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM leupeptin, and 2 µg/mL *N*-acetyl-leucanyl-leucanyl-norleucinal. Lysates were centrifuged for 10 minutes at 4°C in a microcentrifuge, and supernatants were collected for immunoprecipitation. Before immunoprecipitation, cell lysates were subjected to preclearing with non-immune serum and protein A-Sepharose for 15 minutes at 4°C. Equal quantities of each sample (750 µg total protein) were then subjected to immunoprecipitation with anti-PTP1B antibody at 4°C overnight. PTP1B immunocomplexes were precipitated with protein A-Sepharose at 4°C for an additional 2 hours. Immunoprecipitates were washed in PTP assay buffer (100 mM HEPES [pH 7.6], 2 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, and 0.5 mg/mL bovine serum albumin). The peptide RRLIEDAE_pYAARG was added to a final concentration of 200 µM in total reaction volume of 60 µL in PTP assay buffer, and the reaction was allowed to proceed for 1 hour at 30°C. At the end of the reaction, 40-µL aliquots were placed in a 96-well plate, 100 µL malachite green phosphatase reagent was added, and absorbance was measured at 630 nm.

Substrate Trapping In Vitro

Pervanadate stock solution (1 mM) was prepared³³ by adding 10 µL of 100 mM vanadate and 50 µL of 100 mM hydrogen peroxide (diluted from 30% stock in 20 mM HEPES, pH 7.3) to 940 µL H₂O. Excess hydrogen peroxide was removed by adding catalase (100 µg/mL; final concentration, 260 U/mL) 5 minutes after the vanadate and hydrogen peroxide were mixed. Pervanadate solutions were used within 5 minutes to minimize decomposition of the vanadate-hydrogen peroxide complex. IR-transfected HEK-293T cells were treated with 1 mM pervanadate for 30 minutes, washed with phosphate-buffered saline, and lysed in substrate-trapping buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 1% Triton X-100, 150 mM NaCl, 10 mM sodium phosphate, 50 mM sodium fluoride, 5 mM iodoacetic acid, and protease inhibitor mixture [1 mM benzamide, 5 µg/mL leupeptin and 5 µg/mL aprotinin]). Before clarifying the lysates, 10 mM dithiothreitol was added to inactivate any unreacted iodoacetic acid. The lysates were incubated for 2 hours at 4°C with GST or GST-PTP1B-WT or GST-PTP1B-D181A mutant fusion proteins bound to beads. The beads were then washed four times with trapping buffer. Bound proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were then incubated with anti-PY99 or anti-IRβ antibodies and developed by enhanced chemiluminescence (ECL).

Immunoprecipitation

Retinal lysates were solubilized for 30 minutes at 4°C in a lysis buffer (1% Nonidet P-40, 20 mM HEPES [pH 7.4], and 2 mM EDTA) containing

phosphatase inhibitors (100 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM NaVO_3 , and 1 mM molybdate) and protease inhibitors (10 μM leupeptin, 10 $\mu\text{g}/\text{mL}$ aprotinin, and 1 mM PMSF). Insoluble material was removed by centrifugation at 17,000g for 20 minutes, and the solubilized proteins were precleared by incubation with 40 μL protein A-Sepharose for 1 hour at 4°C with mixing. The supernatant was incubated with anti-IR β antibody (4 μg) overnight at 4°C and subsequently with 40 μL protein A-Sepharose for 1 hour at 4°C. After centrifugation at 14,000 rpm for 1 minute, immune complexes were washed three times with wash buffer,³⁴ and the immunoprecipitates were subjected to immunoblot analysis with PY-99 antibody.

SDS-PAGE and Western Blotting

Proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were washed twice for 10 minutes with TTBS (20 mM Tris-HCl [pH 7.4], 100 mM NaCl and 0.1% Tween-20) and were blocked with 5% bovine serum albumin or nonfat dry milk powder (Bio-Rad, Hercules, CA) in TTBS for 1 hour at room temperature. Blots were then incubated with anti-IR β (1:1000) or anti-PTP1B (1:1000) or anti-actin (1:1000) antibodies overnight at 4°C. After primary antibody incubations, immunoblots were incubated with HRP-linked secondary antibodies (mouse or rabbit) and developed by ECL according to the manufacturer's instructions.

Statistical Analysis

All values are expressed as mean \pm SD. Student's *t*-test was used to compare groups. *P* < 0.05 was reported as significant.

RESULTS

Generation and Expression of Substrate-Trapping Mutant of PTP1B

Previously, Flint et al.^{35,36} discovered a mutation of the invariant catalytic acid (Aps-181 in PTP1B) that converts an extremely active enzyme into a "substrate-trap." With the advent of this mutant, several PTP1B substrates have been identified. The cDNA encoding the mouse retinal PTP1B was subjected to site-directed mutagenesis to substitute aspartic acid 181 with alanine. Wild-type and mutant (D181A) PTP1B cDNAs were cloned into pGEX-4T2 vector, and proteins were expressed in *E. coli* and purified to homogeneity. Purified wild-type and mutant GST-PTP1B fusion proteins were run on SDS gels, and PTP1B activity was measured. The results indicate the expression and soluble nature of wild-type and mutant PTP1B fusion proteins (Fig. 1A). Activity measurements clearly indicate that the PTP1B mutant protein failed to catalyze the dephosphorylation of the synthetic peptide compared with the wild-type enzyme (Fig. 1B).

Interaction between PTP1B and IR

To determine whether IR was a physiological substrate of PTP1B, we used the substrate trapping mutant of PTP1B to study the stable interaction between PTP1B and IR. We transiently transfected human IR into HEK-293T cells. The cells were treated with pervanadate before proteins were harvested. Pervanadate-treated cell lysates were incubated with GST, GST-PTP1B, or GST-PTP1B-D181A fusion proteins, and the bound proteins were eluted with sample buffer, fractionated by SDS-PAGE, and immunoblotted with anti-IR β antibody (Fig. 1C). The blot was reprobed with anti-GST antibody to ensure equal amounts of fusion proteins in each pull-down (Figs. 1D, 1E). We observed that the IR specifically bound to PTP1B-D181A mutant from HEK-293T cell lysates but not to GST or wild-type GST-PTP1B (see Fig. 3). The IR is a known substrate of

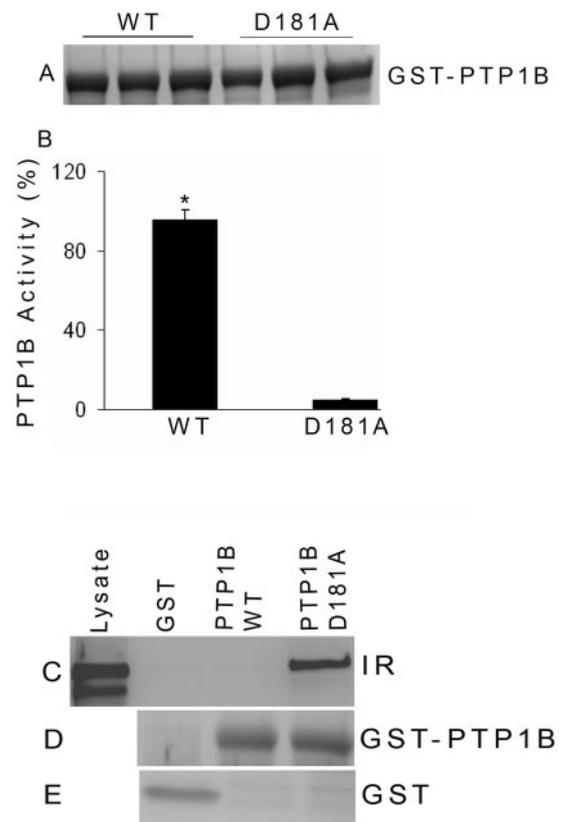


FIGURE 1. Expression and activity of wild-type and mutant PTP1B (D181A). Wild-type and mutant PTP1B GST-fusion proteins were expressed in *E. coli* and purified. The purified proteins were subjected to SDS-PAGE followed by staining (A). PTP1B activity (B) was measured with the use of a PTP1B phosphatase assay kit. To determine the interaction between IR and substrate mutant of PTP1B, human IR was transiently expressed in HEK-293T cells. After 30-minute treatment with pervanadate, cells were lysed and subjected to GST pull-down assay with GST or GST-wild-type PTP1B or mutant PTP1B, followed by immunoblot analysis with anti-IR β antibody (C). The immunoblot was stripped and reprobed with anti-GST antibody to ensure equal amounts of fusion in each lane (D, E).

PTP1B,^{18,37,38} and our results further confirm the interaction between IR and PTP1B.

PTP1B Dephosphorylation of IR Autophosphorylation In Vitro

To determine whether PTP1B dephosphorylates IR autophosphorylation in vitro, we autophosphorylated the cytoplasmic domain of purified human IR (GST-IR) as follows: GST-IR was incubated for 90 minutes at 30°C at 0.05 $\mu\text{g}/\mu\text{L}$ in 25 mM Tris, pH 7.5, 10 mM MgCl_2 , 0.5 mM EGTA, 0.5 mM dithiothreitol, and 0.1 mM ATP.³⁹ Autophosphorylated IR was incubated in the presence of active wild-type PTP1B or catalytically inactive mutant PTP1B for 15 minutes at 30°C. At the end of incubation, SDS sample buffer was added, and the proteins were subjected to immunoblot analysis with anti-PY99 antibody (Fig. 2A). To ensure equal amounts of IR in each reaction, we reprobed the blot with anti-GST antibody (Fig. 2B). Catalytically active and inactive PTP1B in the reaction was also examined on immunoblot with anti-GST antibody (Fig. 2C). The results demonstrate the autophosphorylation of IR in the presence of ATP and that this autophosphorylation was reduced in the presence of wild-type but not mutant PTP1B (Fig. 2A). These

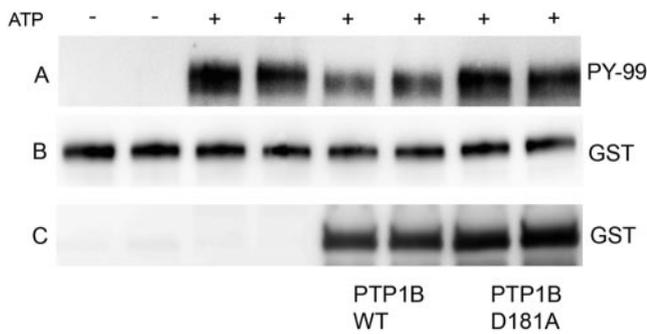


FIGURE 2. In vitro autophosphorylation of the cytoplasmic domain of human IR. GST-IR was incubated for 90 minutes at 30°C in the presence of cold ATP, as described in Results. Autophosphorylated IR (A) was incubated in the presence of wild-type PTP1B or catalytically inactive mutant PTP1B for 15 minutes at 30°C (C). At the end of incubation, SDS sample buffer was added, and the proteins were subjected to immunoblot analysis with anti-PY99 antibody (A). To ensure equal amounts of IR in each reaction, we reprobed the blot with anti-GST antibody (B). Addition of wild-type and mutant PTP1B to the reaction was examined on immunoblot with anti-GST antibody (C).

results suggest that PTP1B dephosphorylates the autophosphorylated IR in vitro.

Localization of PTP1B and IR in the Mouse Retina

Immunohistochemical localization of PTP1B was performed on mouse retinal sections. The results indicate that PTP1B shows more prominent expression in nonphotoreceptor layers (outer plexiform, inner nuclear, and ganglion cell layers) of the retina, though it is clearly expressed in photoreceptors as well (Fig. 3A). Immunohistochemical localization of IR on mouse retinal sections indicates that IR is predominantly expressed in rod inner segments, and weak expression was also observed in outer plexiform, inner plexiform, and ganglion cell layers (Fig. 3B). Omission of primary antibodies of PTP1B or IR did not show any detectable immunostaining (Fig. 3C). Immunohistochemical analysis did not reveal the expression of IR or PTP1B in rod outer segments (ROS). To determine whether PTP1B is expressed in ROS, we prepared ROS from light- and dark-adapted mouse retinas. Light- and dark-adapted ROS proteins were subjected to immunoblot analysis with anti-IR and anti-PTP1B antibodies. The results indicate the expression of IR and PTP1B in ROS, but no difference in expression was observed between light- and dark-adapted ROS membranes (Fig. 3D). Arrestin was used as a positive control for light adaptation, whereas the transducin α -subunit was used as a positive control for dark adaptation. Opsin levels were used as internal

loading control. These observations suggest that IR and PTP1B are expressed in ROS membranes, but its localization is not light dependent.

Autophosphorylation of Retinal IR

IR was immunoprecipitated from rat retina and rat liver with anti-IR β antibody and subjected to in vitro autophosphorylation in the presence and absence of insulin. Phosphorylated samples were resolved by SDS-PAGE, followed by immunoblot analysis with anti-PY99 antibody (Fig. 4A) or subjected to autoradiography (Fig. 4C). The results indicate autophosphorylation of the retinal IR in the absence of insulin, whereas liver IR autophosphorylation was insulin dependent (Figs. 4A, 4C). However, the effect of insulin on retinal IR autophosphorylation is much greater than insulin-induced phosphorylation in the liver. The blot was stripped and reprobed with anti-IR β antibody to ensure equal amounts of receptor were recovered in the immunoprecipitates (Fig. 4B, 4D). These results suggest that retinal IR is constitutively active and that the observed autophosphorylation might have been the result of some other ligand(s) or some unique mechanism that influenced the constitutive autophosphorylation of the IR.

Increased Kinase Activity of Retinal IR in the Absence of Insulin

IR was immunoprecipitated with anti-IR β antibody from lysates of retina and liver, and immune complexes were used to measure the kinase activity in the presence and absence of insulin. In the absence (Fig. 4E) or presence (Fig. 4F) of insulin, retinal IR has high kinase activity compared with IRs of the liver (Figs. 4E, 4F). The increased kinase activity of retinal IR in the presence of insulin suggested that retinal IR has high insulin sensitivity compared with IRs from liver. Results from kinase activity further suggest that the retinal IR is constitutively active and that this activation may be insulin independent.

Reduced IR Autophosphorylation in Hyperglycemia In Vivo

It has previously been shown that IR kinase activity is decreased in diabetes.^{4,40} To determine whether diabetes reduces IR autophosphorylation, we generated hyperglycemic mice with STZ. Mean blood glucose levels of citrate-buffered controls and STZ-injected mice are given in Figure 5A. We considered mice with blood glucose levels higher than 250 mg/dL as hyperglycemic. IR was immunoprecipitated from control and hyperglycemic mice, and the immune complexes were subjected to in vitro autophosphorylation followed by immunoblot analysis with anti-PY99 antibody. The results in-

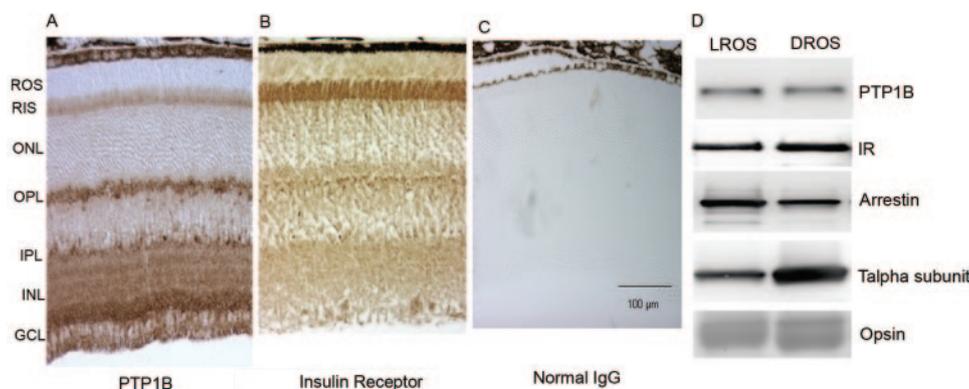


FIGURE 3. Immunocytochemical analysis of PTP1B and IR in mouse retina. Paraffin-fixed sections of rat retina were stained for PTP1B (A), IR (B), or normal IgG (C). ROS, rod outer segments; RIS, rod inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. ROS (20 μ g) prepared from dark- and light-adapted mice were subjected to immunoblot analysis with anti-PTP1B, anti-IR β , anti-arrestin, anti-transducin α ($T\alpha$) subunit and anti-opsin antibodies (D).

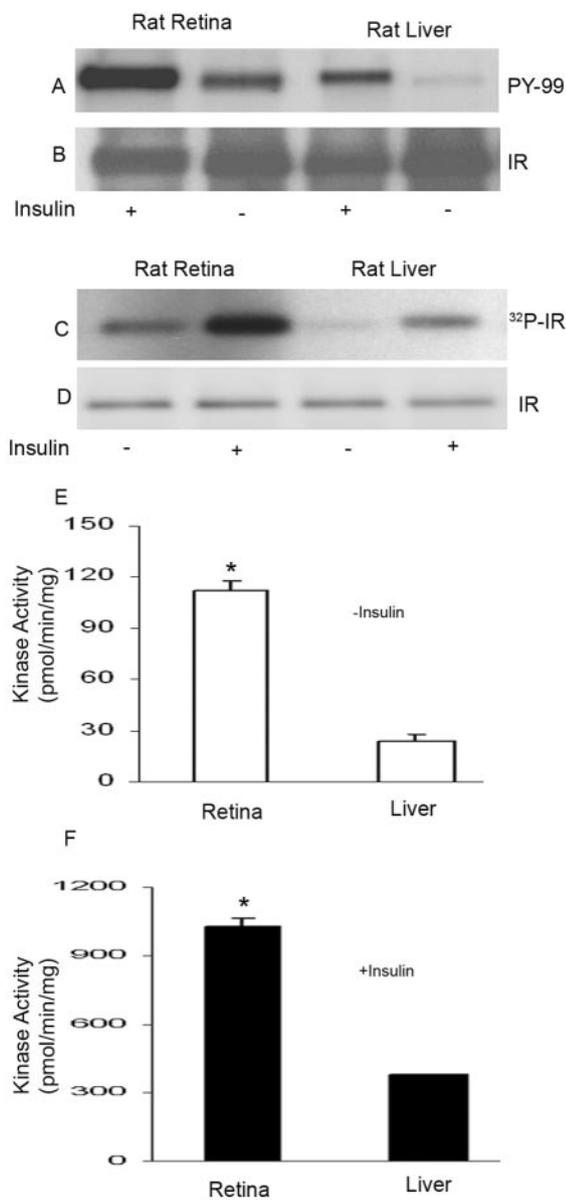


FIGURE 4. Retinal IR displays a high basal level of autophosphorylation. The IR was immunoprecipitated with anti-IR β antibody (B) from rat retinal (500 μ g) and rat liver lysates. Immune complexes were subjected to *in vitro* autophosphorylation (cold or γ ³²p [ATP]) in the presence and absence of 1 μ M insulin. The phosphorylation reaction was subjected to immunoblot analysis with phosphotyrosine (PY-99) antibody (A) or to autoradiography (C). To ensure equal amounts of protein, half the IR immune complexes were subjected to immunoblot analysis with anti-IR β antibody (B, D). Receptor kinase activity was measured in the absence (E) and presence (F) of 1 μ M insulin using poly (Glu-Tyr) substrate in the presence of [γ ³²P]ATP.

dicate decreased retinal IR autophosphorylation in hyperglycemic mice compared with control mice (Fig. 5B). The blot was stripped and reprobed with anti-IR β antibody, and the results indicate no difference in the levels between IR in both groups (Fig. 5C). To determine the efficiency and specificity of IR β antibody in the immunoprecipitation of IR from retinal lysates, we carried out immunoprecipitation with anti-IR β antibody and normal IgG as control. Bound (to normal IgG or IR antibody) and unbound (supernatant after immunoprecipitation) fractions of the immunoprecipitate were subjected to immu-

noblot analysis with anti-IR β antibody, and the results indicate that IR β antibody is able to immunoprecipitate 100% IR but not normal IgG (Fig. 5D). These results demonstrate the specificity and efficiency of IR β antibody. They further indicate that the absence of IR autophosphorylation in diabetic mouse retinas was not caused by incomplete immunoprecipitation.

To further demonstrate whether IR levels are changed by hyperglycemia, we immunoblotted control and STZ retinal lysates with anti-IR β antibody. The results indicate no significant difference in the level of retinal IR between control and hyperglycemic conditions (Figs. 5E, 5F). These results further suggest that retinal IR reduced autophosphorylation in hyperglycemic conditions and that reduced autophosphorylation was not caused by a decrease in the total IR content in the retina.

PTP1B Levels Unchanged in Diabetic Mouse Retinas

To determine whether the state of IR phosphorylation is regulated by PTP1B, we examined PTP1B levels from control and STZ-treated retinas by immunoblot analysis with anti-PTP1B activity. The results indicate no difference in the expression of PTP1B between control and STZ-treated retinas (Fig. 6A). To ensure equal amounts of protein in each lane, we reprobed the immunoblot with anti-actin antibody (Fig. 6B). This experiment demonstrated that retinal PTP1B levels are not increased in diabetes.

Increased PTP1B Activity in Diabetic Mouse Retinas

To determine whether diabetes increases the retinal PTP1B activity, we immunoprecipitated PTP1B with anti-PTP1B antibody and then measured PTP1B activity. These results reveal a significantly increased PTP1B activity in diabetic mouse retinas compared with control retinas (Fig. 7). The PTP1B activity suggests that diabetes increases the retinal PTP1B activity.

DISCUSSION

It has previously been shown that basal IR kinase activity in the retina is significantly greater than in the liver and that IR kinase activity remains constant between freely fed and fasted rats, suggesting that IR activation is not regulated by circulating insulin.^{40,41} The mechanism for high basal levels of IR autophosphorylation and kinase activity is unknown. However, it has been suggested⁴⁰ that the constitutive retinal IR autophosphorylation may be a result of alternative splicing that skips exon 11 to produce a type A receptor.^{42–44} In the liver this exon is included (type B receptor).^{42–44} A significant decrease of IR kinase activity has been reported after 4 weeks of hyperglycemia in STZ-treated rats.⁴ In this study we observed reduced IR autophosphorylation and increased PTP1B activity in hyperglycemic mouse retinas. Several studies have clearly indicated that the state of IR phosphorylation is tightly regulated by PTP1B.^{23,24} Murine models targeting PTP1B show sustained IR phosphorylation in muscle and hepatic tissues,²⁸ increased energy expenditure, and decreased adiposity and tissue-specific insulin sensitivity.²⁷ PTP1B deficiency also reduces insulin resistance and the diabetic phenotype in mice with polygenic insulin resistance.⁴⁵

Our studies indicate that PTP1B and IRs are expressed in rod inner segments. With the use of immunocytochemistry techniques, Rodrigues et al.⁴⁶ found the insulin receptor to be localized in photoreceptor and neuronal cell bodies, with lower immunoreactivity in ROS. The immunohistochemistry of PTP1B shows more prominent expression in nonphotoreceptor layers of the retina, though it is clearly expressed in photoreceptors as well. Our immunohistochemical study on the localization of IR is in agreement with previously reported

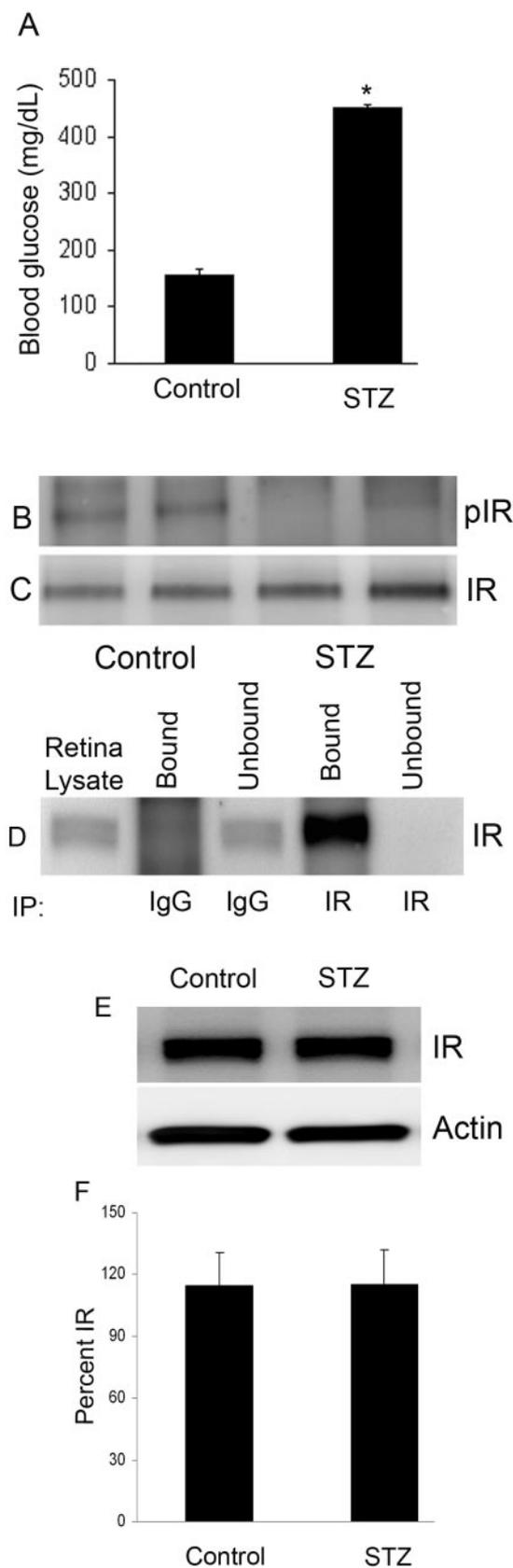


FIGURE 5. Reduced IR autophosphorylation in STZ mouse retinas. Blood was drawn from control and STZ-treated mice from the tail vein, and blood glucose levels were monitored with a glucometer (A). Data are mean \pm SD, $n = 6$. * $P < 0.05$. The IR was immunoprecipitated with

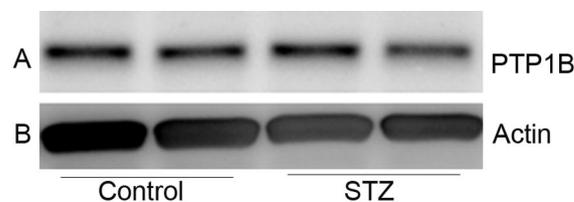


FIGURE 6. PTP1B protein levels in STZ mouse retinas. Twenty micrograms of control and STZ mouse retinal lysates was subjected to immunoblot analysis with anti-PTP1B (A). The PTP1B blot was re-probed with anti-actin (B) antibody to ensure equal amounts of protein in each lane.

studies.⁴⁶ Immunohistochemical analysis did not reveal the expression of PTP1B in the ROS, and this could be attributed to a weak expression in rod outer segments. Rod outer segments prepared from mouse retinas showed the presence of PTP1B in outer segments, but their expression is not light dependent. We have previously reported outer and inner segment localization of IRs in isolated bovine rod photoreceptors.⁴⁷ Furthermore, our recent studies demonstrated that photoreceptor-specific deletion of IRs resulted in stress-induced photoreceptor degeneration.⁶ The colocalization of IR and PTP1B could not be analyzed because IR and PTP1B antibodies are polyclonal. However, the substrate-trap technique used in this study indicated their association, and this interaction might hold true in vivo. Consistent with these observations, the association of IR and PTP1B has previously been shown by FRET analysis.⁴⁸

To determine whether IR is a physiological substrate of PTP1B, we took advantage of a substrate-trapping mutant of PTP1B.³⁵ Substrate-trapping mutants of PTPs are ideal reagents for substrate identification. It was demonstrated that such mutants of PTPs can be produced by the mutation of Asp to Ala in the conserved WPD loop.³⁵ The Asp-to-Ala mutants of PTP1B, TC-PTP, PTPH1, and PTP-PEST helped identify EGFR, p52^{shc}, VCP (p97/CDC48), TYK2 and JAK2, p130^{Cas}, and cortactin as candidate substrates.^{35,36,49–53} The substrate-trapping mutant of PTP1B formed a stable interaction with IR when expressed in HEK-293T cells grown under serum conditions. IR is a known substrate of PTP1B,^{18,37,38} and our results show stable interaction with the substrate-trapping mutant of PTP1B.

A large body of data from cellular, biochemical, mouse, and human genetic and chemical inhibitor studies has identified PTP1B as a major negative regulator of insulin and leptin signaling.^{28,54–57} IR activation has been shown to rescue retinal neurons from apoptosis in culture,⁵ and our recent study clearly indicated the neuroprotective role of IR in photoreceptor survival⁶ because the deletion of IR in rod photoreceptors resulted in stress-induced photoreceptor neurodegeneration. Given that PTP1B regulates the state of IR phosphorylation, it

anti-IR β antibody (B) from retinal lysates (500 μ g) of control and STZ mice, then subjected to in vitro phosphorylation. The reaction was subjected to immunoblot analysis with antiphosphospecific anti-IR (pYpYpY^{1158/1162/1163}) antibody (B). The blot was stripped and re-probed with anti-IR β antibodies (C). Rat retinal lysate was immunoprecipitated with normal IgG (4 μ g) or anti-IR β (4 μ g) antibody, followed by immunoblot analysis of the bound (immune complexes) or the unbound (supernatant after immunoprecipitation) fractions with anti-IR β antibody (D). Twenty micrograms of control and STZ mouse retinal lysates was subjected to immunoblot analysis with anti-IR β and anti-actin (to ensure equal amounts of protein in each lane) antibodies (E). Densitometric analysis of IR immunoblot was performed in the linear range of detection. The control IR was set as 100% (F). Data are mean \pm SD; $n = 4$.

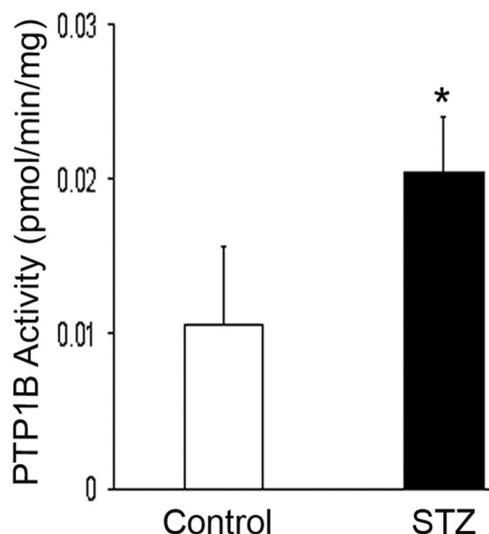


FIGURE 7. PTP1B Activity in STZ mouse retinas. The PTP1B was immunoprecipitated from control and STZ mouse retinas (750 μ g) with anti-PTP1B antibody, and the immune complexes were subjected to PTP1B phosphatase activity. Data are mean \pm SD; $n = 5$. * $P < 0.05$.

is tempting to speculate that PTP1b antagonists are potential therapeutic targets to treat stress-induced retinal degeneration and diabetic retinopathy.

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