

# Transgenic Overexpression of Protein-tyrosine Phosphatase 1B in Muscle Causes Insulin Resistance, but Overexpression with Leukocyte Antigen-related Phosphatase Does Not Additively Impair Insulin Action\*

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Previous studies implicate protein-tyrosine phosphatase 1B (PTP1B) and leukocyte antigen-related phosphatase (LAR) as negative regulators of insulin signaling. The expression and/or activity of PTP1B and LAR are increased in muscle of insulin-resistant rodents and humans. Overexpression of LAR selectively in muscle of transgenic mice causes whole body insulin resistance. To determine whether overexpression of PTP1B also causes insulin resistance, we generated transgenic mice overexpressing human PTP1B selectively in muscle at levels similar to those observed in insulin-resistant humans. Insulin-stimulated insulin receptor (IR) tyrosyl phosphorylation and phosphatidylinositol 3'-kinase activity were impaired by 35% and 40–60% in muscle of PTP1B-overexpressing mice compared with controls. Insulin stimulation of protein kinase C (PKC) $\delta$  activity, which is required for glucose transport, was impaired in muscle of PTP1B-overexpressing mice compared with controls, showing that PTP1B overexpression impairs activation of these PKC isoforms. Furthermore, hyperinsulinemic-euglycemic clamp studies revealed that whole body glucose disposal and muscle glucose uptake were decreased by 40–50% in PTP1B-overexpressing mice. Overexpression of PTP1B or LAR alone in muscle caused similar impairments in insulin action; however, compound overexpression achieved by crossing PTP1B- and LAR-overexpressing mice was not additive. Antibodies against specific IR phosphotyrosines indicated overlapping sites of action of PTP1B

and LAR. Thus, overexpression of PTP1B *in vivo* impairs insulin sensitivity, suggesting that overexpression of PTP1B in muscle of obese humans and rodents may contribute to their insulin resistance. Lack of additive impairment of insulin signaling by PTP1B and LAR suggests that these PTPs have overlapping actions in causing insulin resistance *in vivo*.

A critical regulatory step in insulin signal transduction is the dephosphorylation of signaling molecules by protein-tyrosine phosphatases (PTPs),<sup>1</sup> which participate in terminating insulin signaling. Several PTPs have been implicated in negatively regulating insulin signal transduction, including protein-tyrosine phosphatase 1B (PTP1B) and the leukocyte antigen-related (LAR) phosphatase (1–3). Both LAR and PTP1B are expressed in insulin-responsive tissues such as muscle, liver, and adipose tissue and in brain (1–4). Whereas LAR and PTP1B are both implicated as insulin receptor (IR) and possibly insulin-receptor substrate (IRS) phosphatases (3, 5, 6), multiple PTPs may be required to terminate insulin signaling in different tissues, by acting at discrete subcellular locations, or by targeting distinct tyrosines on the same substrate. In some studies, LAR and PTP1B expression and activity are increased in muscle and adipose tissue of humans and rodents in insulin-resistant states such as obesity (2, 7–11), although other work contradicts these conclusions (12–14). Immunodepletion experiments suggest that a 2- to 3-fold elevation of LAR accounts for most of the enhanced PTP activity in both adipose tissue and muscle of obese humans (9, 15). However, PTP1B polymorphisms in several different populations are also associated with insulin resistance (16–18).

The role of increased expression of PTP1B in insulin resistance has not been evaluated. However, abundant evidence implicates PTP1B in regulating normal insulin action. Crystallographic, kinetic, and peptide binding studies suggest that PTP1B preferentially dephosphorylates double tyrosine motifs such as IR tyrosines 1162–1163 (19), although IRS-1 is also reportedly a substrate for PTP1B *in vitro* (5, 6). In cultured cells, overexpression of PTP1B inhibits insulin signaling (20–

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<sup>1</sup> The abbreviations used are: PTP, protein-tyrosine phosphatase; PTP1B, protein-tyrosine phosphatase 1B; LAR, leukocyte antigen-related phosphatase; IR, insulin receptor; IRS, insulin-receptor substrate; WAT, white adipose tissue; MCK, muscle creatine kinase; MIRKO, muscle-specific insulin receptor-deficient; BAT, brown adipose tissue; PI, phosphoinositide; PKC, protein kinase C.

25). PTP1B-deficient mice (PTP1B<sup>-/-</sup>) have enhanced insulin-stimulated whole body glucose disposal during hyperinsulinemic-euglycemic clamp studies (26). Insulin signaling in skeletal muscle and liver is enhanced in PTP1B<sup>-/-</sup> mice (27). Similarly, insulin action to suppress hepatic glucose output is increased in PTP1B<sup>-/-</sup> mice, and insulin-stimulated glucose uptake is elevated in skeletal muscle but not in white adipose tissue (WAT) (26). PTP1B<sup>-/-</sup> mice also have reduced adiposity and are protected from diet-induced obesity (26, 27). Furthermore, antisense inhibition of PTP1B expression in cultured cells (28) and in mice (29–32) enhances insulin signaling and insulin action.

In cultured cells, LAR can modulate insulin signaling when overexpressed and/or ectopically expressed (33, 34). LAR interacts with IR and inhibits IR tyrosyl phosphorylation (35). *In vitro* studies suggest that LAR preferentially dephosphorylates IR Tyr-1162, one of the three tyrosyl residues that are critical for receptor activity (36). IRS-1 is also a substrate for LAR *in vitro* (5, 6). Conversely, antisense inhibition of LAR gene expression in cultured cells enhances insulin signaling (37–39). The phenotype of LAR-deficient (LAR<sup>-/-</sup>) mice is complex. They have low insulin and glucose levels, and decreased basal hepatic glucose production, consistent with a possible role of LAR as a negative regulator of insulin signaling (40). However, LAR<sup>-/-</sup> mice are also smaller in size and are insulin-resistant in hyperinsulinemic-euglycemic clamp studies, possibly due to the effects of counter-regulatory hormones (40). Nevertheless, transgenic overexpression of LAR in muscle impairs insulin signaling and glucose uptake specifically in muscle and causes whole body insulin resistance (41). Taken together, these studies demonstrate that LAR can negatively regulate insulin signaling and that elevated expression of LAR might contribute to the pathogenesis of insulin resistance *in vivo*.

To determine whether the endogenous overexpression of PTP1B observed in muscle of obese insulin-resistant rodents and humans also contributes to their insulin resistance, we generated mice overexpressing human PTP1B specifically in skeletal muscle. PTP1B-overexpressing mice have decreased insulin-stimulated glucose transport into muscle and whole body insulin resistance, similar to LAR-overexpressing mice (41). Because endogenous overexpression of both LAR and PTP1B occurs in skeletal muscle of obese insulin-resistant rodents and humans, we generated compound muscle-specific LAR- and PTP1B-overexpressing mice to determine whether LAR and PTP1B overexpression additively impair insulin action. Our results reveal that overexpression of LAR and PTP1B does not additively impair insulin action, suggesting that these phosphatases have overlapping actions in causing insulin resistance in muscle.

#### EXPERIMENTAL PROCEDURES

**Transgenic Mice**—The 3.5-kb EcoRI fragment of the human PTP1B cDNA (42) was inserted into an EcoRI site of p3300MCK-CAT (43) (gift of S. Hauschka) downstream of the muscle creatine kinase (MCK) promoter/enhancer start site, and upstream of the SV40 intron and polyadenylation sequences (Fig. 1A). Transgenic MCK-hPTP1B mice were generated by insertion of the NotI-KpnI fragment of the resultant plasmid into the pronuclei of fertilized zygotes from FVB mice. Transgenic mice overexpressing human LAR in muscle (MCK-hLAR) have been previously described (41). Both MCK-hLAR mice and MCK-hPTP1B mice are heterozygous for their respective transgenes and were interbred to generate MCK-hLAR/PTP1B mice. Muscle-specific insulin receptor-deficient (MIRKO) mice (gift of C. R. Kahn) have been previously described (44). Mice were housed at 22 °C with a 12-h light/dark cycle and fed a standard diet (either Purina Autoclavable Rodent Diet 5010 or 5020) *ad libitum*. All studies were approved by the Harvard Medical School Center for Animal Resources and Comparative Medicine and/or Yale Animal Use and Care Committee and were conducted in accordance with the principles and procedures outlined in the

National Institutes of Health Guide for Care and Use of Laboratory Animals.

**PCR Genotyping of Transgenic Mice**—DNA was extracted from mouse toes by proteinase K digestion (45). Human LAR was amplified by PCR using primers hLARpf (5'-CGGGGTGAAGAAGCAAAGAC-3') and hLARpr (5'-CCCTGCCCCATCATCAAAC-3'), which are specific for exon 2. Human PTP1B was amplified by PCR using primers hPTP1Bpf (5'-GTTTCGAGCAGATCGACAAGTC-3') and hPTP1Bpr (5'-TTCTCCATCACTCTGTTGAGC-3'). Primers specific for exon 2 of the cytotoxic T cell lymphocyte-associated 4 gene (5'-TGGTGTGGCTAGCAGCCATG-3' and 5'-TGGGATGGTGAGGTTCACTC-3') were used as amplification controls. PCR reaction conditions are available upon request from J. Zabolotny.

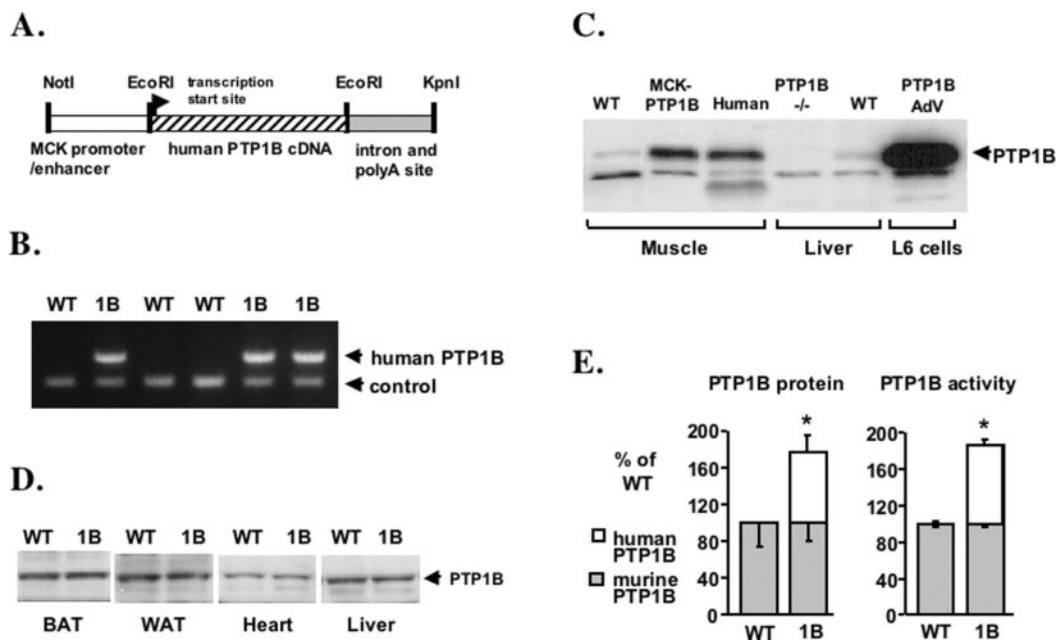
**Measurement of Human and Murine PTP1B Protein in Transgenic Mice**—Liver, heart, interscapular brown adipose tissue (BAT), perigonadal WAT, and hindlimb muscle were dissected from mice and frozen immediately in liquid nitrogen. Tissues were homogenized in 20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 1 min with a Polytron, incubated at 4 °C for 40–60 min with rotation, and centrifuged for 10 min at 16,000 × *g*. Proteins were separated by electrophoresis on 10% SDS-PAGE gels and immunoblotted with antibodies generated against human (23) or murine PTP1B (26). PTP1B was quantified by densitometry (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ) and normalized for cross-reactivity of the anti-human PTP1B antibody for murine PTP1B (6:1, human:mouse) and anti-murine PTP1B antibody for human PTP1B (10:1, murine:human). The relative cross-reactivity was determined by immunoblotting hemagglutinin-tagged human and murine PTP1B expressed in COS cells (data not shown, but see Ref. 23).

**Measurement of Human and Murine PTP1B Activity in Transgenic Mice**—To determine the total amount of PTP1B activity in muscle of MCK-hPTP1B mice, human and murine PTP1B activity against poly-Glu-Tyr was measured in PTP1B immunoprecipitates of WT and MCK-hPTP1B hindlimb skeletal muscle. Murine PTP1B was immunoprecipitated from muscle lysates with polyclonal anti-PTP1B antibodies (26), and human PTP1B was immunoprecipitated with the monoclonal PTP1B antibody FG6 (Calbiochem, San Diego, CA). To determine the specificity of anti-murine PTP1B antibodies for murine PTP1B, PTP1B activity in anti-murine PTP1B immunoprecipitates of WT and MCK-hPTP1B mouse muscle was compared with that of PTP1B<sup>-/-</sup> mouse muscle and human muscle lysates. To determine the cross-reactivity of anti-human PTP1B antibodies for murine PTP1B, PTP1B activity in anti-human PTP1B immunoprecipitates of MCK-hPTP1B muscle lysates was compared with that of WT and PTP1B<sup>-/-</sup> muscle lysates.

**Measurement of Total LAR Protein in Transgenic Mice**—Protein (1 mg) from muscle lysates of WT, MCK-hLAR, MCK-hPTP1B, and MCK-hLAR/PTP1B mice was incubated with wheat germ-agarose overnight, washed three times with lysis buffer, and then bound proteins were separated by SDS-PAGE on 7.5% gels and subjected to anti-LAR immunoblotting as described previously (41).

**Tyrosyl Phosphorylation of IR, IRS-1, and IRS-2**—Fasted (15–18 h) mice were injected intravenously by tail vein with 10 milliuunits of insulin/g of body weight (Humulin, Eli Lilly, Indianapolis IN) then sacrificed 3 min later, and hindlimb muscle and liver were dissected and frozen in liquid nitrogen. Muscle lysates were prepared as described above, and 0.5–1 mg of protein was subjected to immunoprecipitation with anti-phosphotyrosine antibodies (PY99 from Santa Cruz Biotechnology, Santa Cruz, CA, or 4G10 from Upstate Biotechnology, Lake Placid, NY) or anti-IRS-1 or anti-IRS-2 polyclonal antisera (3 µl; gifts from M. White, Joslin Diabetes Center). Immunoconplexes of polyclonal antibodies were collected on protein A-Sepharose (Sigma, St. Louis, MO), and immunoconplexes of monoclonal antibodies were collected on a mix of protein A- and protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). Immune complexes were washed three times with 20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1% Nonidet P-40 and twice with 1× phosphate-buffered saline (Invitrogen, Gaithersburg, MD) with 2 mM Na<sub>3</sub>VO<sub>4</sub>, resuspended in SDS-PAGE sample buffer, and heated for 5 min at 95 °C. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, incubated with anti-phosphotyrosine monoclonal antibodies (PY99, Santa Cruz Biotechnology, or 4G10, Upstate Biotechnology) and visualized using chemiluminescence. With this protocol, the efficiency of immunoprecipitation of IRS-1 is ≥95% and IRS-2 is ≥90% (data not shown).

Muscle lysates were immunoblotted with polyclonal antibodies against the IR β subunit (gift of C. R. Kahn, Joslin Diabetes Center), phosphotyrosines 972, 1158, or 1162–1163 of IR (BIOSOURCE Inter-



**FIG. 1. MCK-PTP1B transgenic mice have low level PTP1B overexpression specifically in muscle.** *A*, schematic diagram of the human PTP1B transgene. The muscle creatine kinase (*MCK*) promoter and enhancer region, human PTP1B cDNA, and SV40 intron and polyadenylation sequences are represented by *open*, *stippled*, and *gray boxes*, respectively. Relevant restriction sites and the transcriptional start site are shown. *B*, human PTP1B-overexpressing mice were genotyped by PCR. Amplified DNA from wild-type (*WT*) or PTP1B-overexpressing (*1B*) mice was electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized with ultraviolet illumination. Amplified DNA corresponding to the human PTP1B transgene and control cytotoxic T cell lymphocyte-associated 4 gene is indicated with *arrows*. *C*, transgenic mice overexpressing PTP1B in muscle were identified by immunoblotting. Lysates of muscle from wild-type (*WT*) or muscle-specific human PTP1B-overexpressing mice, or from normal human muscle, PTP1B<sup>-/-</sup> liver, *WT* liver, and L6 cells infected with human PTP1B-expressing adenovirus (*AdV*) as a positive control were made, proteins were separated by SDS-PAGE and immunoblotted with antibodies against human PTP1B. *D*, transgenic mice do not overexpress PTP1B in other tissues. Proteins in lysates of BAT, WAT, liver, or heart from wild-type (*WT*) or muscle-specific human PTP1B-overexpressing (*1B*) mice were separated by SDS-PAGE and immunoblotted with antibodies against human PTP1B. PTP1B levels from several *WT* and MCK-PTP1B mice were quantified by densitometry and were not different. *E*, PTP1B protein (*left*) and activity (*right*) levels in muscle of *WT* and PTP1B-overexpressing (*1B*) mice. Human and murine PTP1B proteins were detected by immunoblotting with species-specific antibodies generated against human and mouse isoforms of PTP1B. Immunoblots were quantified by densitometry and normalized for cross-reactivity of anti-human PTP1B antibody for murine PTP1B and anti-murine PTP1B antibody for human PTP1B (see “Experimental Procedures”). PTP1B activity in anti-human and anti-murine PTP1B immunoprecipitates of *WT* and MCK-PTP1B muscle lysates against poly-Glu-Tyr was measured.

national, Camarillo, CA), IRS-1 and IRS-2 (gifts of M. White, Joslin Diabetes Center) (46), or ERK1/2 (gift of J. Blenis, Harvard Medical School) following the manufacturer's directions or standard techniques. Phosphorylation of specific IR tyrosines and total ERK1/2 were visualized with fluorescent secondary antibodies and quantified with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), following the manufacturer's instructions. For all other experiments, proteins were visualized by chemiluminescence and quantified by densitometry (Molecular Dynamics/Amersham Biosciences).

**PI 3'-Kinase, PKC $\zeta$ , and Akt Activity Assays**—Tissue lysates (250–1000  $\mu$ g of protein) were subjected to immunoprecipitations with 3  $\mu$ l of IRS-1 or IRS-2 polyclonal antibodies (1:100 dilution, gift of M. White, Joslin Diabetes Center) or 2  $\mu$ g of monoclonal anti-phosphotyrosine antibody (PY99 from Santa Cruz Biotechnology) for PI 3'-kinase assays. Immune complexes were collected on protein A- (Sigma) or protein G-Sepharose (Amersham Biosciences) and washed, and PI 3-kinase activity was measured as reported previously (47). For Akt activity measurements, muscle lysates (500  $\mu$ g of protein) were immunoprecipitated for 4 h at 4 °C with 3  $\mu$ g of polyclonal Akt antibodies that recognize Akt1 and Akt2 (Upstate Biotechnology, Lake Placid, NY) coupled to protein G-Sepharose beads (Amersham Biosciences). The immune complexes were washed, and Akt activity was determined as previously described (47). For measurement of protein kinase C (PKC) isoforms  $\lambda$  and  $\zeta$  activity, muscle lysates (250  $\mu$ g of protein) were subjected to immunoprecipitation for 4 h at 4 °C with 2  $\mu$ g of a polyclonal anti-PKC antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognizing both PKC $\lambda$  and PKC $\zeta$ , coupled to protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ). Immune pellets were washed, and PKC  $\lambda/\zeta$  activity was determined as previously described (48).

**Measurement of Glucose, Insulin, and Free Fatty Acid Levels**—Blood was extracted from the tail veins of fed or overnight fasted (16–18 h) mice. For hyperinsulinemic-euglycemic clamp studies, plasma glucose was measured by the glucose oxidase reaction using a Beckman glucose

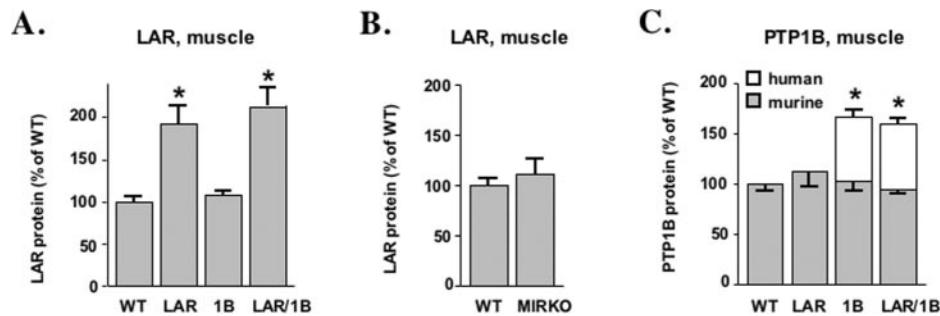
analyzer II (Beckman, Fullerton, CA). For all other studies, blood glucose levels were measured using a One Touch II glucometer (LifeScan Inc., Johnson & Johnson, Milpitas, CA). For hyperinsulinemic-euglycemic clamp studies, plasma insulin levels were determined by radioimmunoassay (Linco Research, St. Charles, MO). For all other experiments, serum insulin levels were determined with a rat insulin enzyme-linked immunosorbent assay (Crystal Chem Inc., Chicago, IL) using mouse insulin standards.

**Hyperinsulinemic-Euglycemic Clamps**—Hyperinsulinemic-euglycemic (103  $\pm$  9 mg/dl) clamp studies were performed as described previously (41, 49) on male mice 5 months of age. Insulin was infused continuously for 120 min at 2.5 milliunits/kg/min. Hepatic glucose production and muscle 2-deoxyglucose uptake, glycolysis and glycogen synthesis were measured as described (41, 49).

**Statistical Analyses**—Data are presented as means  $\pm$  S.E. Statistical analyses were performed using StatView software (Abacus Concepts, Inc., Berkeley, CA). Statistical significance was tested with unpaired Student's *t* tests or analysis of variance.

## RESULTS

**Generation of Muscle-specific Human PTP1B (MCK-hPTP1B)-overexpressing Mice**—To determine if endogenous PTP1B overexpression, as observed in muscle of insulin-resistant humans and rodents (7–9), could cause insulin resistance, we generated mice that overexpress human PTP1B selectively in muscle (Fig. 1, *A–E*). PTP1B overexpression in skeletal muscle was 1.8-fold over *WT* as determined by immunoblotting and PTP1B activity assay (Fig. 1*E*). Overexpression of human PTP1B did not alter the level of murine PTP1B protein or murine PTP1B activity (Fig. 1*E*). The level of transgenic PTP1B overexpression observed in MCK-hPTP1B mice is similar to that observed in skeletal muscle of insulin-resistant

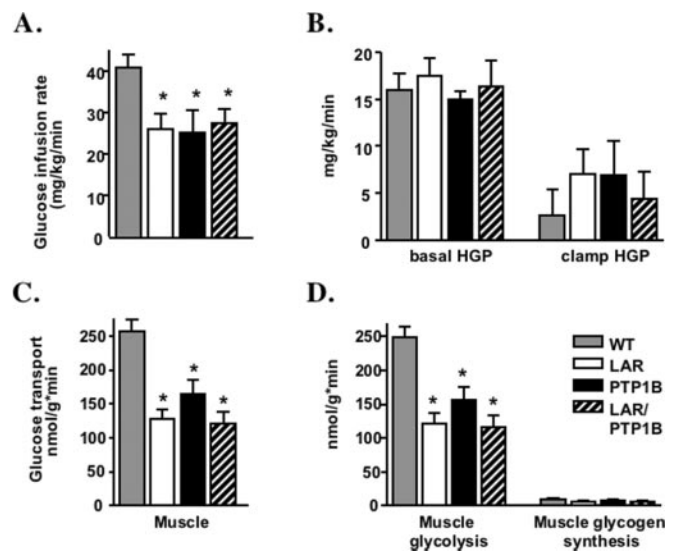


**FIG. 2. Overexpression of PTP1B and LAR in muscle of transgenic mice.** LAR protein levels in muscle of wild-type (WT), MCK-hLAR (LAR), MCK-hPTP1B (1B), and MCK-hLAR/PTP1B (LAR/1B) mice (A) and WT and muscle-specific insulin receptor-deficient (MIRKO) mice (B). Human and murine LAR proteins from muscle lysates were detected by immunoblotting with an antibody generated against human LAR. Immunoblots were quantified by densitometry and normalized for the differential affinity of anti-human LAR antibody for murine and human LAR. PTP1B protein levels in muscle of WT, MCK-hLAR, MCK-hPTP1B, and MCK-hLAR/PTP1B mice (C). Human and murine PTP1B proteins from muscle lysates were detected by immunoblotting with species-specific antibodies generated against human and mouse isoforms of PTP1B. Immunoblots were quantified by densitometry and normalized for cross-reactivity of anti-human PTP1B antibody for murine PTP1B and anti-murine PTP1B antibody for human PTP1B.

humans and rodents (7–9). No overexpression of PTP1B was detected in brown adipose tissue (BAT), white adipose tissue (WAT), heart, or liver (Fig. 1D).

**Transgenic Overexpression of PTP1B in Muscle Causes Whole Body Insulin Resistance Similar to LAR Overexpression**—We previously showed that muscle-specific human LAR-overexpressing (MCK-hLAR) mice have decreased whole body glucose disposal rates and muscle glucose uptake, indicating that overexpression of LAR in muscle causes insulin resistance (41). To determine whether compound overexpression of PTP1B and LAR additively or synergistically impairs insulin action, we crossed MCK-hPTP1B and MCK-hLAR mice to obtain transgenic mice overexpressing both PTP1B and LAR in muscle (MCK-hLAR/PTP1B). To verify that in MCK-hLAR/PTP1B mice, overexpression of PTP1B in muscle was similar to MCK-PTP1B mice and overexpression of LAR in muscle was similar to MCK-hLAR mice, we measured human and murine PTP1B protein and total LAR protein in muscle of WT, MCK-hLAR, MCK-hPTP1B, and MCK-hLAR/PTP1B mice. To avoid potential differential expression of the transgenes in different muscle fiber types, determinations of PTP1B and LAR expression and the effects thereof were made in mixed fiber type hindlimb skeletal muscles, including gastrocnemius and tibialis. Endogenous PTP1B levels were similar in muscles of WT, MCK-hLAR, MCK-hPTP1B, and MCK-hLAR/PTP1B mice (Fig. 2C). Levels of the human PTP1B protein were also similar in muscles of MCK-hPTP1B and MCK-hLAR/PTP1B mice (Fig. 2C). Total LAR levels were similar in muscle of WT and MCK-hPTP1B mice and increased to a similar extent in muscles of MCK-hLAR and MCK-hLAR/PTP1B mice (Fig. 2A). In addition, LAR expression was similar in muscles of WT and muscle insulin receptor-deficient (MIRKO) mice (Fig. 2B), an animal model in which insulin signaling is absent in muscle, suggesting that insulin resistance in muscle does not alter LAR expression. Thus, overexpression of PTP1B does not change expression of LAR, and *vice versa*.

Hyperinsulinemic-euglycemic clamp studies (26, 41, 49) showed that the glucose infusion rate needed to maintain euglycemia in MCK-hPTP1B mice was decreased by 37% compared with WT, indicating that MCK-hPTP1B mice are insulin-resistant (Fig. 3A). A similar impairment (37%) in glucose uptake was seen in MCK-hLAR mice (Fig. 3A), as reported in our previous study (41). The glucose infusion rate was also decreased by 36% in MCK-hLAR/PTP1B mice (Fig. 3A), indicating that compound overexpression of LAR and PTP1B in skeletal muscle does not additively impair insulin action. Basal hepatic glucose production and the action of insulin to suppress hepatic glucose production were normal in MCK-hPTP1B,



**FIG. 3. Muscle-specific PTP1B-overexpressing mice have whole body and muscle insulin resistance similar to muscle-specific LAR-overexpressing mice.** Hyperinsulinemic-euglycemic clamp studies of male wild-type (WT), MCK-hLAR (LAR), MCK-hPTP1B (PTP1B), and MCK-hLAR/PTP1B (LAR/PTP1B) overexpressing mice. Glucose infusion rate (A), basal and clamp hepatic glucose production (HGP) (B), insulin-stimulated glucose transport into skeletal muscle (C), and muscle glycolysis and muscle glycogen synthesis (D) in WT, LAR, PTP1B, and LAR/PTP1B-overexpressing mice are shown. Results are means  $\pm$  S.E. for  $n = 6-8$  per genotype; \*,  $p \leq 0.05$  compared with WT.

MCK-hLAR, and MCK-hLAR/PTP1B mice (Fig. 3B). Insulin-stimulated glucose transport (Fig. 3C) and glycolysis (Fig. 3D) in skeletal muscle were impaired in MCK-hPTP1B, MCK-hLAR, and MCK-hLAR/PTP1B mice by 40–50% compared with WT mice, indicating that PTP overexpression in muscle impaired insulin action in muscle. Muscle glycogen synthesis also tended to be decreased (Fig. 3D).

Insulin resistance in muscle can result in increased body fat, as observed in mice deficient in IR specifically in muscle (44). However, body weight and perigonadal fat pad weight of male (Table I) and female (data not shown) MCK-hPTP1B mice fed a standard chow diet were not different from WT controls. Similarly, body weight and perigonadal fat pad weight of MCK-hLAR mice on standard chow diets were not different from WT littermates (Table I), as previously reported (41), and adiposity was also normal in MCK-hLAR/PTP1B mice. Blood glucose and serum insulin from both overnight fasted and random fed animals was similar in WT and all transgenic mice (Table I).

TABLE I  
Metabolic characteristics of wild-type and muscle-specific LAR-, PTP1B-, and LAR/PTP1B-overexpressing mice

Data are from male mice in the fed (7 months) or overnight fasted (9 months) states.  $n = 6-9$  per genotype. All parameters were also normal in mice at younger ages.

Genotype	WT	LAR	PTP1B	LAR/PTP1B
Body weight (g)	32.0 ± 0.4	31.4 ± 0.4	31.2 ± 0.9	33.7 ± 1.0
Fed glucose (mg/dl)	110 ± 4	122 ± 3	119 ± 4	118 ± 3
Fed insulin (ng/ml)	2.1 ± 0.3	1.7 ± 0.2	2.0 ± 0.2	2.5 ± 0.2
Fasted glucose (mg/dl)	67.1 ± 3.1	70.9 ± 4.9	80.1 ± 6.5	76.3 ± 6.4
Fasted insulin (ng/ml)	0.46 ± 0.08	0.36 ± 0.09	0.54 ± 0.07	0.40 ± 0.13

**Compound Overexpression of LAR and PTP1B Does Not Additively Impair Insulin Signaling in Muscle *in Vivo***—To determine why compound overexpression of LAR and PTP1B does not additively impair insulin-stimulated glucose uptake into muscle and whole body glucose homeostasis, we examined insulin signal transduction in skeletal muscle of MCK-hPTP1B, MCK-hLAR, and MCK-hLAR/PTP1B mice compared with WT. Insulin-stimulated total tyrosyl phosphorylation of IR was decreased by 35% in muscle of MCK-hPTP1B mice (Fig. 4A). In contrast, insulin-stimulated total tyrosyl phosphorylation of IR was not decreased in MCK-hLAR mice compared with WT (Fig. 4A), as previously reported (41). Total IR tyrosyl phosphorylation was impaired in MCK-hLAR/PTP1B mice (Fig. 4A), to a similar extent as in MCK-hPTP1B mice. We reported previously that total tyrosyl phosphorylation of IRS-2 was reduced upon insulin stimulation in muscle of MCK-hLAR mice compared with WT, but total tyrosyl phosphorylation of IRS-1 was unchanged (41). Tyrosine phosphorylation was detected using anti-phosphotyrosine antibody PY99. However, using a different anti-phosphotyrosine antibody to detect tyrosine phosphorylation of IRS-1 (4G10), insulin-stimulated tyrosyl phosphorylation of IRS-1 and IRS-2 was decreased to similar extents (34–58%) in muscle of MCK-hPTP1B, MCK-hLAR, and MCK-hLAR/PTP1B mice compared with WT controls (Fig. 4, B and C). There was a tendency toward lower insulin-stimulated IRS-2 tyrosyl phosphorylation in muscle of MCK-hLAR mice compared with MCK-hPTP1B mice, and lower insulin-stimulated IRS-1 tyrosyl phosphorylation in muscle of MCK-hPTP1B mice compared with MCK-hLAR mice, although neither of these differences between the genotypes reached statistical significance. Levels of total IR, IRS-1, and IRS-2 were similar in muscle of WT and all transgenic mice (data not shown). Insulin-stimulated phosphotyrosine-, IRS-1, and IRS-2-associated PI 3'-kinase activity all were decreased by 31–60% in muscle of MCK-hPTP1B, MCK-hLAR, and MCK-hLAR/PTP1B mice compared with WT (Fig. 4, D–F). Insulin-stimulated Akt activity in muscle was similar in WT and all transgenic mice (Fig. 4G). In contrast, insulin-stimulated PKC $\zeta$  activity was impaired by ~40% in muscle of MCK-hLAR, MCK-hPTP1B, and MCK-hLAR/PTP1B (Fig. 4H).

Assays described above were performed using female mice, and similar signaling impairments are observed in male MCK-hLAR mice, as we previously reported (41). Insulin-stimulated phosphotyrosine- and IRS-1-associated PI 3-kinase activity in muscle of male MCK-hPTP1B mice was impaired by 42% ( $100 \pm 12.8$  for WT and  $58.5 \pm 9.7$  for PTP1B,  $p \leq 0.05$ ) and 30% ( $100 \pm 8.2$  for WT and  $69.3 \pm 5.7$  for PTP1B,  $p \leq 0.05$ ), respectively, compared with WT mice. This is similar to that observed in female MCK-hPTP1B mice (Fig. 4D), indicating that there are no gender-specific differences in insulin action in contrast to those reported for PTP1B-deficient mice (26). Gender-specific differences in PTP1B-deficient mice may be due to the influence of different genetic backgrounds or the effects of PTP1B in tissues other than muscle.

To determine whether overexpression of PTP1B in muscle altered insulin action in other insulin-target tissues, we exam-

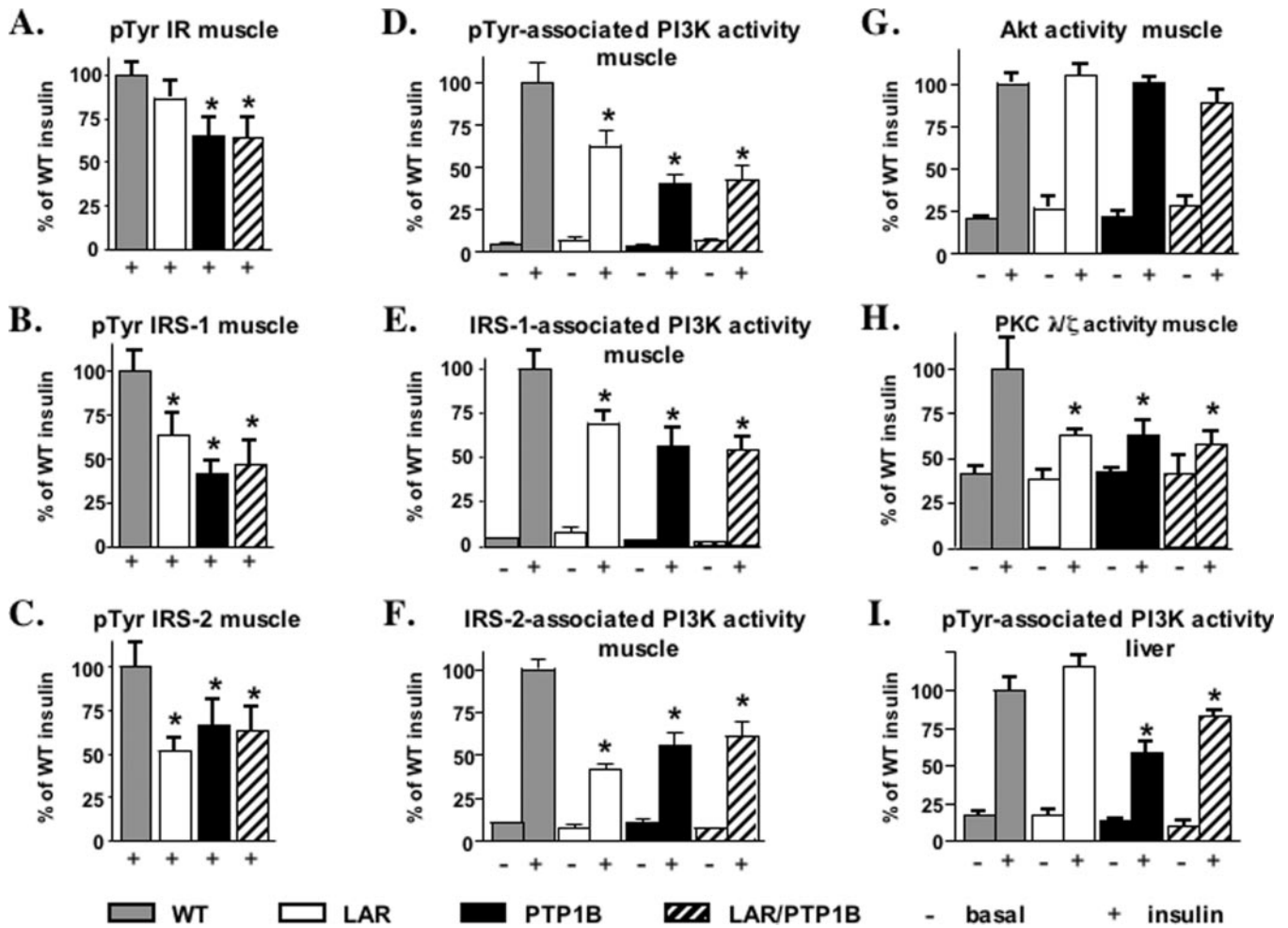
ined insulin signaling in liver of MCK-hPTP1B mice. We found no defects in insulin-stimulated tyrosyl phosphorylation of IR or IRS-1 in liver (data not shown). However, insulin-stimulated phosphotyrosine-associated PI 3'-kinase activity in liver was decreased by 41% in MCK-hPTP1B mice and by 17% in MCK-hLAR/PTP1B mice, but not MCK-hLAR mice compared with WT (Fig. 4I). Similar results were obtained in four separate experiments and were independent of gender or age of animals. Hyperinsulinemic-euglycemic clamp studies did not reveal any defect in the suppression of hepatic glucose production of insulin (Fig. 3B) in PTP1B-overexpressing mice.

**LAR and PTP1B Have Overlapping Actions as IR Phosphatases**—To determine why compound overexpression of LAR and PTP1B did not additively impair insulin signaling in muscle, we examined insulin-stimulated phosphorylation of specific insulin receptor tyrosines using antibodies specific for phosphotyrosines 972, 1158, or 1162–1163 of IR or the insulin-like growth factor 1 receptor. Importantly, in MIRKO mice, we did not detect any insulin-stimulated phosphorylation of insulin-like growth factor 1 receptor in muscle (data not shown). Thus, under these conditions, these phospho-specific antibodies appear to be specific for IR in muscle. In MCK-hPTP1B, MCK-hLAR, and MCK-hLAR/PTP1B mice, insulin-stimulated tyrosine phosphorylation of IR pY<sup>1162–1163</sup> was decreased by 22–35% in muscle compared with WT (Fig. 5C), consistent with previous reports that LAR (36) and PTP1B (19) preferentially dephosphorylate one or both of the tyrosines of this double tyrosine motif of IR. Similar decreases in tyrosine phosphorylation of Tyr-972 and Tyr-1158 were also observed in muscle of MCK-hPTP1B, MCK-hLAR, and MCK-hLAR/PTP1B mice compared with WT controls (Fig. 5, A and B), suggesting that LAR and PTP1B have overlapping specificities as IR phosphatases, at least when overexpressed.

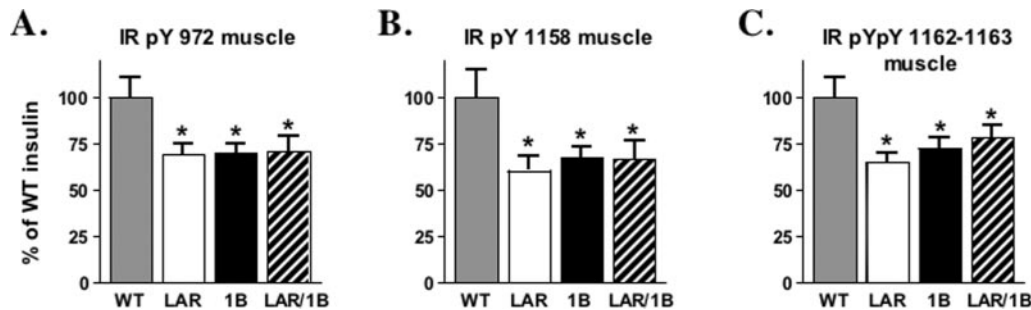
## DISCUSSION

Our data demonstrate that overexpression of PTP1B in muscle *in vivo* impairs insulin signaling in muscle and results in whole body insulin resistance. This suggests that the endogenous 2–2.5-fold PTP1B overexpression seen in muscle of obese humans and rodents (7–9) may contribute to the pathogenesis of insulin resistance. Our data also demonstrate that overexpression of LAR and PTP1B in muscle do not additively impair insulin action. This may be because, when overexpressed in muscle, LAR and PTP1B dephosphorylate the same IR phosphotyrosines. Thus, our data suggest that when LAR and PTP1B are concurrently overexpressed in muscle and adipose tissue as occurs in obese insulin-resistant humans and rodents (2, 7–11), these phosphatases have overlapping actions in negatively regulating IR phosphorylation and insulin action.

PTP1B deficiency in mice has been shown to cause increased insulin sensitivity (26, 27). Now, our data establish that even low level PTP1B overexpression *in vivo* causes insulin resistance. Insulin resistance in muscle can increase body fat, as observed in mice deficient in IR specifically in muscle (44). However, our data show no significant increase in body weight of muscle-specific PTP1B-overexpressing mice, most likely be-



**FIG. 4. Insulin signaling is impaired in muscle of muscle-specific PTP1B-overexpressing mice similar to muscle-specific LAR-overexpressing mice.** Female wild type (WT), MCK-hLAR (LAR), MCK-hPTP1B (PTP1B), and MCK-hLAR/PTP1B (LAR/PTP1B)-overexpressing mice were injected intravenously with saline (-) or 10 milliunits/g insulin (+) and sacrificed 3 min later. Phosphotyrosine (A)-, IRS-1 (B)-, or IRS-2 (C)-associated immunoprecipitates from muscle lysates were separated by SDS-PAGE and immunoblotted for phosphotyrosine (pTyr), and bands corresponding to IR, IRS-1, or IRS-2 were quantified by densitometry. PI 3-kinase activity of phosphotyrosine (D)-, IRS-1 (E)-, or IRS-2 (F)-associated immunoprecipitates from muscle lysates and phosphotyrosine-associated immunoprecipitates from liver lysates (I), Akt activity in muscle against Crosstide substrate (G), and PKC $\lambda/\zeta$  activity (H) were measured. Results are means  $\pm$  S.E. for  $n = 1-3$  per genotype for basal and  $n = 5-8$  per genotype for insulin-stimulated conditions; \*,  $p \leq 0.05$  compared with WT.



**FIG. 5. LAR and PTP1B dephosphorylate the same IR phosphotyrosines when overexpressed in muscle.** Tyrosine phosphorylation of IR in muscle of PTP-overexpressing mice. Female wild-type (WT), MCK-hLAR (LAR), MCK-hPTP1B (1B), and MCK-hLAR/PTP1B (LAR/1B)-overexpressing mice were injected intravenously with saline or 10 milliunits/g insulin and sacrificed 3 min later. Muscle lysates were separated by SDS-PAGE, immunoblotted for IR phosphotyrosines 972 (A), 1158 (B), or 1162-1163 (C), and bands corresponding to phosphorylated IR were quantified by an Odyssey scanner (LI-COR Biosciences). Results are means of phosphorylated IR normalized to the levels of ERK1 and ERK2. Results are means  $\pm$  S.E. for  $n = 7$  per genotype; \*,  $p \leq 0.05$  compared with WT.

cause PTP1B overexpression reduces but does not obliterate insulin signaling, as does IR deficiency (44).

Although overexpression of both PTP1B and LAR has been shown to negatively regulate insulin signaling, our data show that overexpression of PTP1B or LAR does not impair insulin-stimulated Akt activity in muscle. This is similar to what has been observed in muscle of humans with type II diabetes (50).

In contrast, overexpression of PTP1B or LAR impairs insulin-stimulated PKC $\lambda/\zeta$  activity in muscle. Several studies suggest that activation of atypical PKC isoforms  $\lambda$  and  $\zeta$  is required for insulin stimulation of glucose uptake and glucose transporter 4 translocation in muscle and adipocytes (51-54). Insulin-stimulated PKC $\lambda/\zeta$  activity is impaired in muscle and adipose tissue of insulin-resistant animals (55-58) and humans (59-62). Our

data suggest that the endogenous overexpression of PTP1B and LAR that occurs in muscle and adipose tissue of insulin-resistant animals and humans (2, 7–11) may impair glucose transport in these tissues by negatively regulating insulin-stimulated PKC $\zeta$  activity.

Interestingly, PTP1B overexpression in muscle decreased insulin-stimulated PI 3-kinase activity in the liver of PTP1B-overexpressing mice. The fact that there is no defect in insulin-stimulated IR or IRS tyrosyl phosphorylation in liver supports this being an indirect effect. Several recent studies utilizing tissue-specific gene targeting to ablate genes regulating insulin action have revealed intercommunication among insulin-target tissues, presumably through secreted factors (63). Insulin-stimulated PI 3-kinase activity modulates expression of phosphoenolpyruvate carboxykinase and other genes that regulate hepatic gluconeogenesis (64). The discrepancy between impaired insulin-stimulated PI 3-kinase activity in liver and normal insulin-stimulated suppression of hepatic glucose production may reflect 1) the different dose and duration of insulin used in the two experiments, 2) that the defect in PI 3-kinase activity is not sufficiently severe to alter hepatic gluconeogenesis, or 3) that other pathways contribute to regulation of insulin action on hepatic gluconeogenesis. Impaired insulin-stimulated PI 3-kinase activity in liver of a number of insulin-resistant animal models (65–71) likely contributes to insulin resistance in liver and hyperinsulinemia and/or hyperglycemia. However, failure of insulin to suppress hepatic glucose production in high fat-fed (72), high sucrose-fed (73), and high salt-fed (74, 75) animal models is accompanied by increased insulin-stimulated PI 3-kinase activity in liver, supporting the notion that other pathways can regulate hepatic gluconeogenesis *in vivo*.

Our results clearly demonstrate that compound overexpression of LAR and PTP1B does not additively impair insulin signaling or action *in vivo*. Overexpression of LAR in muscle does not affect expression of endogenous PTP1B or the human PTP1B transgene in muscle. Similarly, overexpression of PTP1B in muscle does not affect expression of the LAR transgene. In addition, endogenous levels of LAR are similar in muscle of WT and insulin-resistant MIRKO muscle, indicating that insulin resistance itself does not alter LAR expression. We cannot exclude the possibility that overexpression of either PTP regulates the activity of the other PTP. However, altered LAR or PTP1B activity is unlikely to account for the lack of additive action of LAR and PTP1B on insulin action in MCK-hLAR/PTP1B mice for the following reasons. If overexpression of LAR negatively regulates PTP1B activity, this negative regulation would be expected to occur in the MCK-hLAR mice as well as the MCK-hLAR/PTP1B mice. Due to the very low level of LAR overexpression, and the fact that reduced PTP1B activity causes increased insulin sensitivity, an insulin-resistant phenotype such as we have observed in the MCK-hLAR mice would be unlikely. Similar reasoning would apply if PTP1B overexpression regulates LAR activity, particularly because the level of PTP1B overexpression is less than 2-fold in MCK-hPTP1B mice.

In previous *in vitro* studies, LAR reportedly exhibited specificity for dephosphorylating IR tyrosine 1162 (36). Reports differ on the relative specificity of PTP1B for insulin receptor phosphotyrosines. Crystallographic, kinetic, and peptide binding studies suggest that PTP1B prefers double phosphotyrosine motifs such as IR 1162–1163 (19). However, other studies dispute this notion. For example, binding to IR of a catalytically inactive mutant of PTP1B is blocked with similar concentrations of phosphopeptides of the IR kinase domain (tyrosines 1158–1163), NPXY motif (tyrosine 972), or C terminus (76).

Our data demonstrate that, when overexpressed, LAR and PTP1B diminish phosphorylation of the same IR tyrosyl residues *in vivo*. LAR or PTP1B may directly dephosphorylate IR tyrosines 972, 1158, and 1162–1163. Alternatively, they may directly dephosphorylate only tyrosines 1162–1163, which would decrease the kinase activity of IR and lead to decreased phosphorylation of other IR tyrosines. Regardless of whether the two PTPs directly dephosphorylate the same sites *in vivo*, the net effect of overexpression of either PTP on IR phosphorylation is the same.

Surprisingly, we did not detect decreased insulin-stimulated total IR tyrosyl phosphorylation in LAR overexpressors, most likely due to differential affinity of “total anti-phosphotyrosine” antibodies for different phosphotyrosine residues. PTP1B has been reported to target phosphotyrosines in the IR C terminus in addition to the NPXY and kinase domain phosphotyrosines (76). Thus, the difference in total IR tyrosyl phosphorylation in muscle of LAR *versus* PTP1B-overexpressing mice may be due to the actions of PTP1B on phosphotyrosines other than those of the NPXY and kinase domains. Because LAR and PTP1B each dephosphorylate tyrosines critical to IR kinase activity and substrate binding ability, the effects of LAR and PTP1B overexpression are not additive *in vivo*.

Notwithstanding this, the lack of additivity of LAR and PTP1B overexpression on negatively regulating insulin action is unexpected, because overexpression of either LAR or PTP1B does not reduce IR or IRS tyrosine phosphorylation to basal levels. It is not clear why additive impairments in insulin action do not occur with compound overexpression of LAR and PTP1B. Localization or trafficking of IR may be altered through interaction with one of these PTPs, which may limit its access to the other PTP. The magnitude of PTP overexpression achieved in both LAR- and PTP1B-overexpressing mice is similar to endogenous LAR and PTP1B overexpression observed in muscle and adipose tissue of obese insulin-resistant humans (7–9). Thus, although enhanced negative regulation of insulin action may be achieved with greater PTP overexpression, our data are informative because the level of transgenic overexpression achieved reflects endogenous overexpression occurring in obese insulin-resistant states. Thus, even low level PTP overexpression has a significant effect on insulin resistance in muscle. Lack of additive negative regulation of LAR and PTP1B overexpression on insulin action *in vivo* indicates further study is needed to illuminate the roles of different PTPs in regulating insulin action *in vivo*.

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