PTEN Expression Contributes to the Regulation of Muscle Protein Degradation in Diabetes

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OBJECTIVE—Conditions accelerating muscle proteolysis are frequently associated with defective phosphatidylinositol 3-kinase (PI3K)/Akt signaling and reduced PI3K-generated phosphatidylinositol 3,4,5-triphosphate (PIP_3). We evaluated the control of muscle protein synthesis and degradation in mouse models of type 1 and 2 diabetes to determine whether defects besides PI3K/Akt activities affect muscle metabolism.

RESEARCH DESIGN AND METHODS—We evaluated the expression and activity of PTEN, the phosphatase converting PIP_3 to inactive phosphatidylinositol 4,5-bisphosphate, and studied how PTEN influences muscle protein in diabetic wild-type mice and in mice with partial deficiency of $PTEN^{+/-}$.

RESULTS—In acutely diabetic mice, muscle PTEN expression was decreased. It was increased by chronic diabetes or insulin resistance. In cultured C2C12 myotubes, acute suppression of PI3K activity led to decreased PTEN expression, while palmitic acid increased PTEN in myotubes in a p38-dependent fashion. To examine whether PTEN affects muscle protein turnover, we studied primary myotubes cultures from wild-type and $PTEN^{+/-}$ mice. The proteolysis induced by serum deprivation was suppressed in $PTEN^{+/-}$ cells. Moreover, the sizes of muscle fibers in $PTEN^{+/-}$ and wild-type mice were similar, but the increase in muscle proteolysis caused by acute diabetes was significantly suppressed by $PTEN^{+/-}$. This antiproteolytic response involved higher PIP_3 and *p*-Akt levels and a decrease in caspase-3–mediated actin cleavage and activation of the ubiquitin-proteasome system as signified by reduced induction of atrogin-1/MAFbx or MurF1 (muscle-specific RING finger protein 1).

CONCLUSIONS—Changes in PTEN expression participate in the regulation of muscle proteolytic pathways. A decrease in PTEN could be a compensatory mechanism to prevent muscle protein losses. ***Diabetes* 56:2449–2456, 2007**

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DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FOXO, forkhead transcription factor; IRS, insulin receptor substrate; MurF1, muscle-specific RING finger protein 1; *p*-Akt, phosphorylated Akt; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-triphosphate; STZ, streptozotocin; TLC, thin-layer chromatography; UPS, ubiquitin-proteasome system.

Many catabolic conditions including diabetes are associated with progressive muscle atrophy, which contributes to the morbidity of these conditions. The mechanism for atrophy could involve decreased protein synthesis, although a major anabolic influence of insulin is to depress protein degradation in muscle (1–3). Virtually all of the conditions causing muscle protein losses are associated with activation of the ubiquitin-proteasome system (UPS) to degrade proteins (4,5). In fact, muscle proteolysis depends on two critical events: first, cleavage of actomyosin/myofibrils by caspase-3, yielding substrates for the UPS (3,6–8). Second, there is increased expression of E3 ubiquitin ligases atrogin-1/MAFbx and muscle-specific RING finger protein (MurF1), which is associated with increased activity of the UPS (9,10). The presence of the characteristic 14-kDa actin fragment in muscle of animal models of diabetes and in patients with kidney failure, atrophy associated with disuse, or burn injury is evidence for initial cleavage of actomyosin as a triggering event in muscle protein loss (3,6,7,11).

Experimental evidence indicates that muscle proteolysis in catabolic conditions is linked to insulin resistance and, specifically, to defects in the insulin receptor substrate (IRS)-1–associated phosphatidylinositol 3-kinase (PI3K)/Akt pathway (3,7,12,13). Defective PI3K/Akt signaling reduces the level of the PI3K-generated product, phosphatidylinositol 3,4,5-triphosphate (PIP_3), with a subsequent decrease in Akt activation (*p*-Akt). This in turn leads to activation of both caspase-3 and forkhead transcription factors (FOXOs). The former leads to actomyosin cleavage, while the latter response increases atrogin-1 expression and stimulates the breakdown of muscle proteins in the UPS.

Another mechanism that could lower PIP_3 levels is a reciprocal change in the activity of PTEN (the phosphatase and tensin homolog deleted from chromosome 10) because it dephosphorylates PIP_3 to form inactive phosphatidylinositol 4,5-bisphosphate. In short, a rise in PTEN activity should have the same impact on insulin/IGF-1 signaling as a decrease in PI3K activity (14,15). The possibility that PTEN might participate in controlling muscle protein metabolism is raised because PTEN can vary in catabolic conditions; for example, insulin resistance induced by a high-fat diet increases PTEN activity (16). Moreover, PTEN can influence cellular metabolism because skeletal muscle–specific deletion of PTEN improves glucose homeostasis in mice (17). During development, cells with decreased PTEN activity are larger (18), and cardiomyocytes engineered to inactivate PTEN undergo hypertrophy (19). Finally, the development of cancer is associated with an increase in *p*-Akt and loss of

PTEN (20). The influence of variations in PTEN and the control of muscle protein metabolism has received limited attention.

We found that PTEN expression in muscle varies in models of defects in insulin signaling. In acute type 1 diabetes, PTEN in muscle is reduced. In chronic models (e.g., in *db/db* mice with insulin resistance or in mice 4 months after streptozotocin-induced diabetes), both PTEN mRNA and protein are upregulated in muscle. In cultured muscle cells, we found that changes in PI3K and p38 activity are involved in the regulation of the PTEN level. To examine whether PTEN expression changes muscle metabolism, we studied mice with partial deletion of the PTEN gene and evaluated muscle protein synthesis and degradation. Our results indicate that a lower PTEN activity, as occurs in acute diabetes, prevents muscle loss even when IRS-1–associated PI3K activity is low and unchanging. The initial decrease in PTEN could be a compensation to maintain protein balance in muscle.

RESEARCH DESIGN AND METHODS

Animal models of diabetes. Experiments were approved by the Baylor College of Medicine Animal Care Committee. *db/db* mice aged 10 weeks (The Jackson Laboratory), models of insulin resistance, were compared with littermate, wild-type mice. Four months after 65 mg streptozotocin (STZ)/kg was injected intraperitoneally on 2 consecutive days, long-term diabetes was assessed in comparison with control age-matched C57B6 mice. The acute type 1 diabetic mice were injected intraperitoneally with 150 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} STZ on 2 days and 5 days later were compared with control mice injected with the diluent and pair fed against the STZ-injected mice (2,7). Diabetes was confirmed by glucosuria (Urine Strip; Fisher, Fairlawn, NJ). We also studied PTEN$^{+/-}$ mice and pair-fed littermate wild-type mice before and after STZ injection (2,21).

Primary cell culture, PTEN, and protein degradation. C2C12 myoblasts (ATCC; Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS). At 70% confluence, the media was changed to DMEM plus 2% horse serum and resulting myotubes treated with 2% horse serum and BSA or horse serum plus 0.4 mmol/l palmitate in BSA. We also examined C2C12 myotubes treated with 0.4 mmol/l palmitate with or without expression of a dominant-negative p38 produced by mutagenesis of two phosphorylation sites of p38α (180T/A and 182Y/F). Other C2C12 cells were treated with 10% FBS with or without the PI3K inhibitor, 10 μmol/l LY294002 (Calbiochem, San Diego, CA). After 0, 3, 6, and 12 h, cells were harvested for analyses of proteins or mRNAs.

Primary myoblasts were isolated from hindlimb muscles of PTEN$^{+/-}$ and littermate wild-type mice as described by Rando and Blau (22). Growth media consisted of 1:1 DMEM:F10 media, 10% FBS, 5 ng/ml bFGF, 200 units/ml penicillin G, and 200 μg/ml streptomycin. The rate of protein degradation in these cells was calculated from the L-[U-^{14}C]phenylalanine released from prelabeled cells as previously described (23).

Muscle histology and protein synthesis and degradation. Arterial blood from anesthetized (20 mg/kg ketamine; 4 mg/kg xylazine) mice was used to measure glucose. Tibialis anterior muscles were used to evaluate total protein content, and soleus and extensor digitorum longus muscles were used to determine protein synthesis and degradation. The lateral gastrocnemius muscle was immediately frozen in liquid nitrogen and stored at −80°C; the medial gastrocnemius was fixed at resting length and imbedded in HistoPrep Frozen Embedding Media (Fisher, Pittsburgh, PA), and 5-μm sections were stained for laminin or caspase-3. To determine the cross-sectional area, we used Image Pro software (Silver Spring, MD) and assessed 600 muscle cells in each section. Caspase-3 activity in muscle was detected by immunofluorescence using an anti–cleaved caspase-3 antibody (Cell Signaling, Beverly, MA) followed by Alex 488-secondary antibody (7). Myofibers were outlined by laminin staining, and nuclei were visualized by DAPI. The negative control was normal rabbit lgG instead of the primary antibody; autofluorescence was suppressed using autofluorescence eliminator (Chemicon, Temecula, CA). The percentage of caspase-3–positive fibers in a cross-section of the entire middle gastrocnemius muscle was calculated for each of six mice in each group. To measure the total protein content, the entire tibialis anterior muscle was grinded in 0.5 N NaOH; after complete dissolution, protein concentration was determined by the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Protein synthesis in isolated, incubated muscles was measured as the incorporation of L-[U-^{14}C]phenylalanine, while protein degradation was evaluated from the release of tyrosine (24).

Cell signaling in muscle analyzed by immunoblotting. The lateral gastrocnemius muscle (~50 mg) was homogenized in 0.5 ml lysis buffer (50 mmol/l Tris [pH 7.4]; 1% NP-40; 0.25% Na-deoxycholate; 150 mmol/l NaCl; 1 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mmol/l Na_3VO_4; and 1 mmol/l NaF). After centrifugation (16,000*g*) at 4°C for 15 min, supernatant proteins were analyzed by Western blotting using the following antibodies: *p*-Akt (Thr437), *p*-FOXO, *p*-p38, and PTEN (Cell Signaling, Beverly, MA), as well as Akt1, FOXO1, p38, and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA). The 14-kDa actin fragment as another index of muscle proteolysis was measured as described (6,7). Densities were determined with an infrared laser scanner (Odyssey LiCor, Lincoln, NB).

RNA preparation, Northern blot, and quantitative real-time PCR analyses. Gastrocnemius muscle RNA was isolated (TRI reagent; Sigma-Aldrich, St. Louis, MO), and 20 μg RNA was separated on a 1% agarose gel, transferred to a nylon transfer membrane (Amersham Pharmacia Biotech, Piscataway, NJ), and ultraviolet cross-linked. Hybridization was performed (Quick Hybrid; Stratagene, La Jolla, CA) for 2 h at 64°C before washing the blot twice with 2× and once with 0.5× SSC (saline sodium citrate buffer) (1× SSC: 0.15 mol/l NaCl plus 0.015 mol/l sodium citrate), containing 0.1% sodium dodecyl sulfate (7). α-^{32}P dCTP–labeled atrogin-1, MurF1, or PTEN cDNA probes were synthesized using a random primer labeling kit (Amersham) and purified with G-25 columns (Roche, Indianapolis, IN). Real-time PCR was performed as described using 2 μg total RNA and the following primers: mouse PTEN, forward 5′-AATTCCCAGTCAGAGGCGCTATGT-3′ and reverse 5′-GATTGCAA GTTCCGCCACTGAA CA-3′, and mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase), forward 5′-ACCCCCAATGTATCCGTTGT-3′ and reverse 5′-TACTCCTTGGAGGCCATGTA-3′ (3).

PI3K and PTEN activities. The lateral gastrocnemius muscle (~50 mg) was homogenized for 5 min in 1 ml buffer (137 mmol/l NaCl, 20 mmol/l Tris-HCl [pH 7.4], 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 0.1 mmol/l Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride, and 1% Nonidet-P40) and kept on ice for 15 min before centrifuging at 13,000*g* for 10 min. Lysate (2 mg) was immunoprecipitated with 2 μg anti–IRS-1 antibody overnight at 4°C (3). Immunoprecipitates were washed three times with buffer and three times with TNE buffer (20 mmol/l Tris-HCl [pH 7.4], 100 mmol/l NaCl, and 0.5 mmol/l EGTA). We measured PI3K activity as described, and ^{32}P-labeled inositol lipids were visualized and analyzed by autoradiography using NIH Image J (7).

PTEN activity was measured by immunoprecipitating 600 μg muscle lysates with 20 μl anti-PTEN antibody overnight, followed by incubation with protein A/G-agarose for 1 h at 4°C. Precipitates were washed five times with lysate buffer containing 10 μm dithiothreitol. The enzyme activity was measured using an enzyme-linked immunosorbent assay kit (Echelon, Salt Lake City, UT).

Measurement of PIP_3 in muscle. PIP_3 in ~100 mg fresh gastrocnemius muscle was measured by quickly homogenizing it in 1 ml 1:1 methanol: chloroform plus 50 μl 12 N HCl. After centrifugation (16,000*g* for 10 min), the organic phase was dried under vacuum, the residue was suspended in 40 μl chloroform, and the phospholipids were separated on activated thin-layer chromatography (TLC) plates (Sigma-Aldrich) using 7:10:1.5:2.5 chloroform: methanol:ammonium hydroxide:water over 3 h (25). Minutes after intravenously injecting insulin (150 μg/kg), muscle PIP_3 was measured as a positive control. After drying, TLC plates were treated with Odyssey blocking solution for 30 min and probed overnight with an anti-PIP_3 monoclonal antibody (Echelon). IR dye-800–labeled rabbit anti-mouse lgG antibody (Li-Cor) was incubated with TLC plates for 1 h at room temperature and scanned with the Odyssey system.

Statistical analysis. Results are means ± SE. Differences between two groups were analyzed by the *t* test; multiple comparisons were analyzed by ANOVA with a post hoc analysis by the Student-Newman-Keuls test for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

PTEN expression in skeletal muscle varies in different models of diabetes. In atrophying muscles from mice studied 5 days after STZ-induced insulin deficiency as a model of type 1 diabetes, PTEN protein levels were ~50% lower than those in muscle of control mice (Fig. 1*A*). There was no change in PTEN mRNA, suggesting that acute insulin deficiency involves posttranslational modification of PTEN (Fig. 1*B*). Similar results occurred in

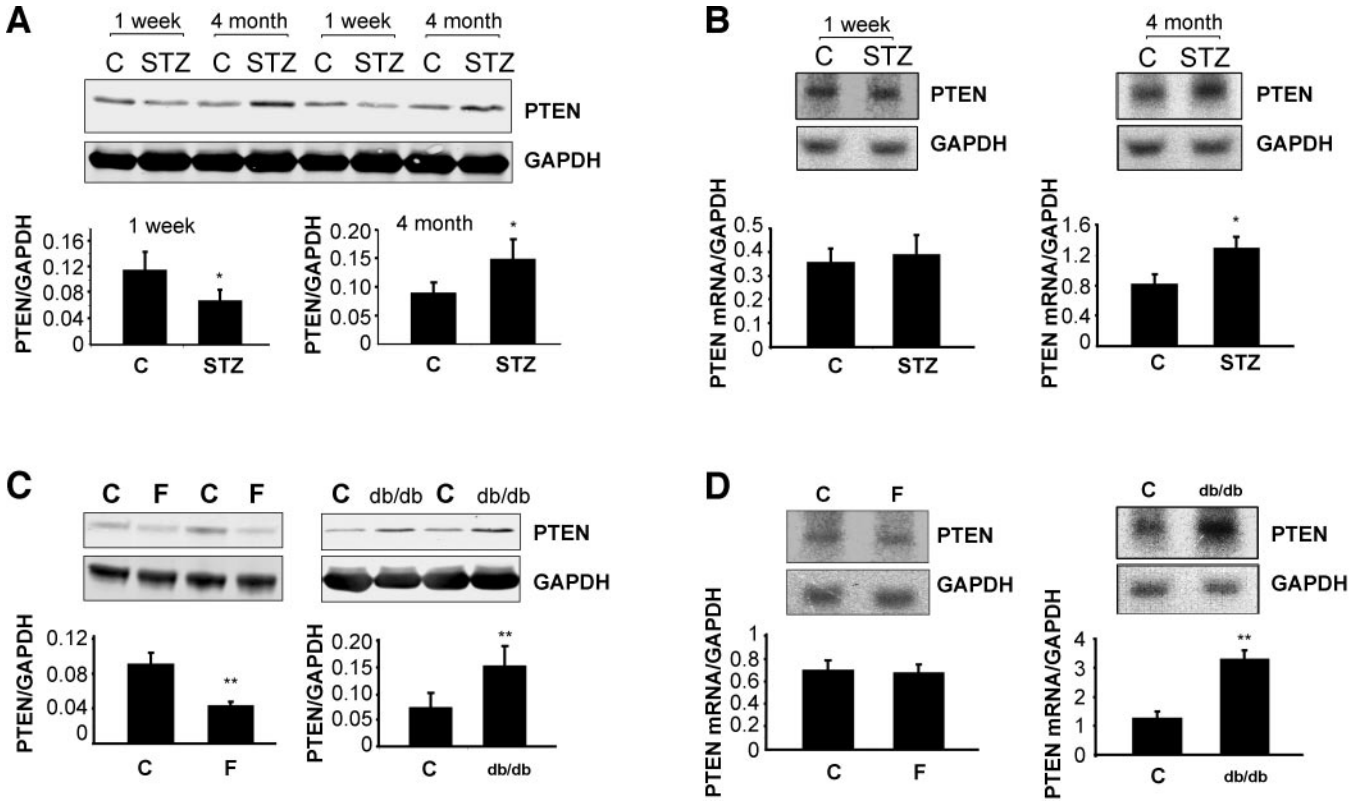

FIG. 1. PTEN expression in skeletal muscle varies with the type of diabetes. Muscle PTEN protein and mRNA levels were examined by Western (*A*) and Northern (*B*) blots; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control (C). Results from mice with acute (1 week) and long-term (4 months) diabetes are shown. Western (*C*) and Northern (*D*) blots of PTEN in muscle of fasted (F) and insulin-resistant (*db/db*) mice are shown. Statistical analyses of results from four long-term diabetic mice and six mice in each of the other groups are shown in the bar graphs (P* < 0.05; ***P* < 0.01).**

muscle of mice fasted for 24 h (Fig. 1*C* and *D*), indicating that results in acute diabetes were not simply a toxic response to STZ.

With prolonged abnormalities in insulin signaling (e.g., models of type 2 diabetes or long-term STZ injection), muscle PTEN expression differed markedly from responses to acute diabetes. There was a 2.5-fold increase in PTEN in muscle of insulin-resistant *db/db* mice and a 60% increase in muscle of mice injected 4 months earlier with STZ. In addition, PTEN mRNA was increased threefold in muscle of *db/db* mice and ~50% in mice with long-term diabetes (Fig. 1*B* and *D*). These changes suggest that PTEN is transcriptionally regulated in chronic insulin deficiency/resistance.

Wang et al. (26) reported that p38 can influence PTEN expression; we therefore evaluated changes in the p38 pathway. As shown in Fig. 2*A*, the level of phosphorylated p38 in muscle was not significantly changed in response to acute insulin deficiency or fasting but was increased in muscle of *db/db* mice and mice with long-term STZ-induced diabetes. To explore mechanisms for these differences, we incubated C2C12 myotubes with palmitic acid and found that it increased the level of activated p38 and levels of PTEN protein and mRNA. We also incubated C2C12 muscle cells with palmitic acid in the presence of dominant-negative p38; p38 inhibition blocked the increase in PTEN mRNA and protein (Fig. 2*B* and *C*).

Since acute diabetes or fasting is associated with decreased PI3K activity (7), we treated C2C12 cells with a specific PI3K inhibitor, LY294002 (10 μmol/l), and measured PTEN. There was a rapid decrease in PTEN over 6 h and a return toward control levels by 12 h (Fig. 2*D*). Note that there was no change in *p*-p38 or the mRNA of PTEN over 12 h, consistent with findings in vivo (Fig. 2*E*). These results suggest that downregulated PTEN in acute diabetes involves posttranslational mechanisms, while PTEN upregulation in chronic diabetes involves transcriptional mechanisms.

PTEN and suppression of protein catabolism in differentiated muscle cells. To determine the physiologic relevance of changes in PTEN expression on muscle proteolysis, we established primary cultures of muscle cells from wild-type mice and mice with partial PTEN deficiency. The cells were differentiated into myotubes by incubating in 2% horse serum DMEM. We stimulated proteolysis by serum withdrawal and measured the rate of protein degradation over 24 h. Protein degradation rates in wild-type and PTEN$^{+/-}$ myotubes incubated in 2% horse serum were not different. However, when proteolysis was stimulated by serum starvation, the increase in protein degradation occurring in wild-type cells was significantly ($P < 0.05$) reduced in PTEN$^{+/-}$ myotubes (Fig. 3*A*).

To examine the mechanism by which a decrease in PTEN could protect against excessive muscle protein losses, we measured *p*-Akt because we and others have shown that low *p*-Akt activity in muscle is associated with an increase in atrogin-1/MAFbx expression and protein degradation (7,27,28). *p*-Akt was not different in PTEN$^{+/-}$ or wild-type myotubes cultured in 2% horse serum. But with serum starvation, *p*-Akt in PTEN$^{+/-}$ myotubes was higher than in wild-type muscle cells (Fig. 3*B*). We also assessed the expression of atrogin-1/MAFbx. Serum starvation sig-

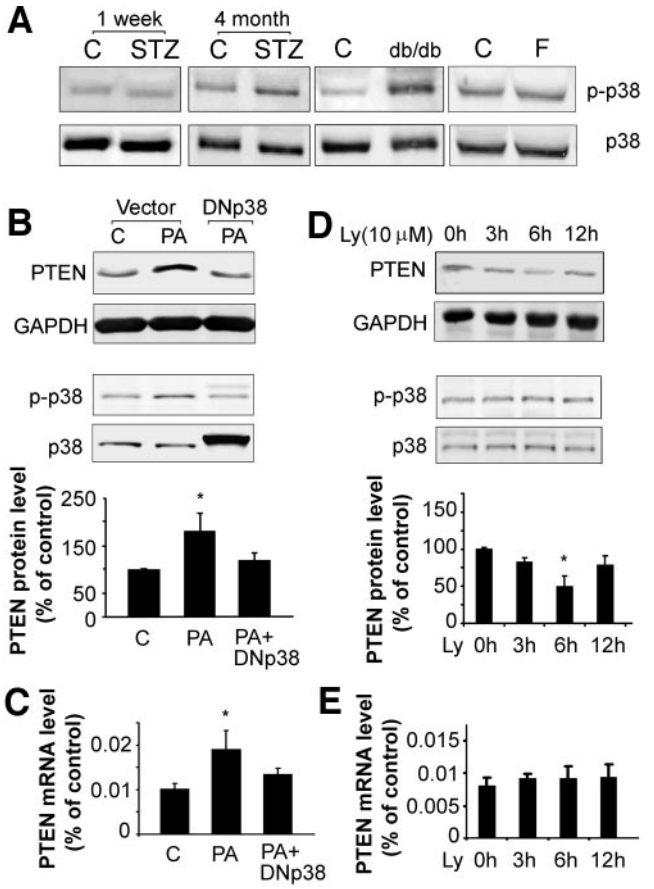

FIG. 2. PTEN upregulation in muscle of mice with long-term diabetes is linked to p38 mitogen-activated protein kinase. *A*: Phosphorylation of p38 in muscles of acutely (1 week) diabetic, long-term (4 months) diabetic, db/db, and fasted (F) mice is shown. C, control treatment. *B*: C2C12 myotubes were treated with 0.4 mmol/l palmitic acid (PA) for 24 h in the presence or absence of dominant-negative p38; PTEN and phosphorylated p38 proteins were assessed by Western blot. A representative blot is shown in the *upper panel*, and results from three independent experiments are summarized in the bar graph (*$P < 0.05$). *C*: Real-time PCR analyses of PTEN expression in C2C12 myotubes treated as described in *B*. The PTEN mRNA level was increased by palmitic acid (*$P < 0.05$), and this response was blocked by the dominant-negative p38. *D*: The influence of PI3K activity on PTEN expression was determined by incubating C2C12 myotubes from 0 to 12 h with 10 μmol/l LY294002, a PI3K inhibitor. The PTEN level and the phosphorylation of p38 were determined by Western blotting (*upper panels*). Representative blots are shown, and results from three independent experiments are summarized in the bar graph (*$P < 0.05$). *E*: Real-time PCR analyses of PTEN expression in C2C12 myotubes treated as described in *D*. Inhibition of PI3K activity did not change PTEN mRNA levels.

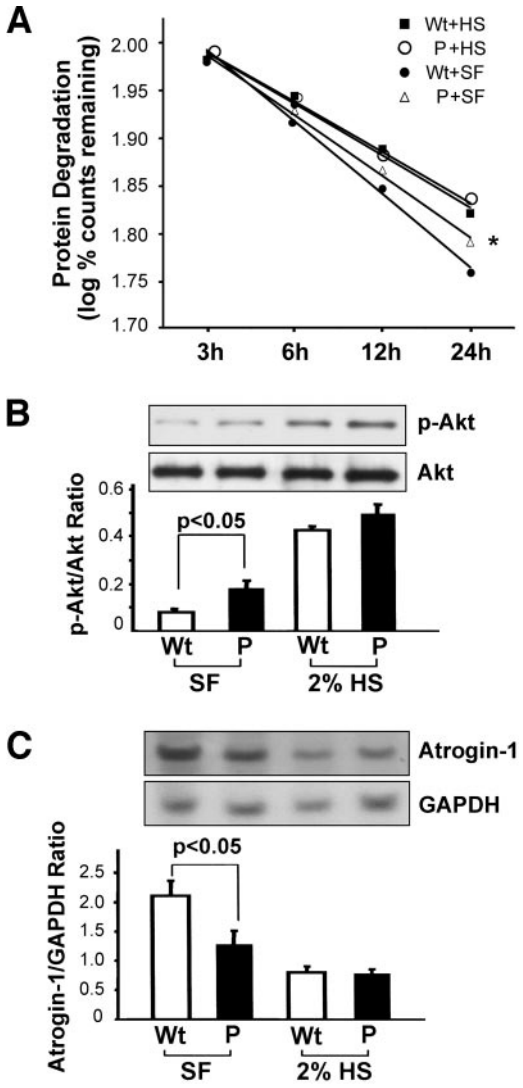

FIG. 3. PTEN partial deletion suppresses protein degradation in primary cultures of myocytes. *A*: The increase in protein degradation was suppressed in myotubes from mice with partial deletion of PTEN. Protein degradation rates were calculated from the release of L-[U-^{14}C] phenylalanine from prelabeled cells. The basal protein degradation rates measured in wild-type (Wt) and PTEN$^{+/-}$ (P) myotubes with 2% horse serum (HS) were unchanged. When myotubes were serum starved (SF), the increase in protein degradation was blunted in PTEN$^{+/-}$ cells [PTEN$^{+/-}$ plus serum-starved myotubes] (*$P < 0.05$ vs. rate in wild-type plus serum-starved myotubes; $n = 6$). *B*: The suppression of proteolysis was associated with an increase in *p*-Akt. Cells were serum starved for 24 h, and the levels of *p*-Akt and total Akt were measured by Western blotting. *C*: mRNA from myotubes cultured in serum-starved or horse serum–supplemented media was used in a Northern blot to determine atrogin-1/MAFbx mRNA expression; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

nificantly increased atrogin-1 mRNA expression in both types of cultured myotubes, but the increase was significantly less in cells from PTEN$^{+/-}$ mice than in those from wild-type mice (Fig. 3*C*).

PTEN regulates muscle protein degradation in vivo. Decreased IRS-1–associated PI3K activity increases muscle protein degradation in catabolic conditions including insulin resistance and acute insulin deficiency (2,3,21, 27,29). To determine whether reduced PTEN expression can counteract muscle protein catabolism in vivo, we minimized changes in PI3K by injecting a high dose of STZ into wild-type and PTEN$^{+/-}$ mice. After 5 days, the blood glucose in STZ-injected mice was approximately sixfold higher than in wild-type mice (33.5 ± 2.8 vs. 5.2 ± 0.7 mmol/l, respectively), and the islet damage in both types of mice was similar (data not shown). Without STZ injection, the cross-sectional area of muscle fibers and the total muscle protein from pair-fed wild-type and PTEN$^{+/-}$ mice did not differ. With acute insulin deficiency, the muscle cross-sectional area and protein content in wild-type mice were significantly less than those in pair-fed PTEN$^{+/-}$ mice (Fig. 4*A* and *B*).

We examined whether changes in protein synthesis or degradation in red-fiber soleus or white-fiber extensor digitorum longus could account for the muscle loss. The rates of protein synthesis in each type of muscle from diabetic wild-type and PTEN$^{+/-}$ mice were not different

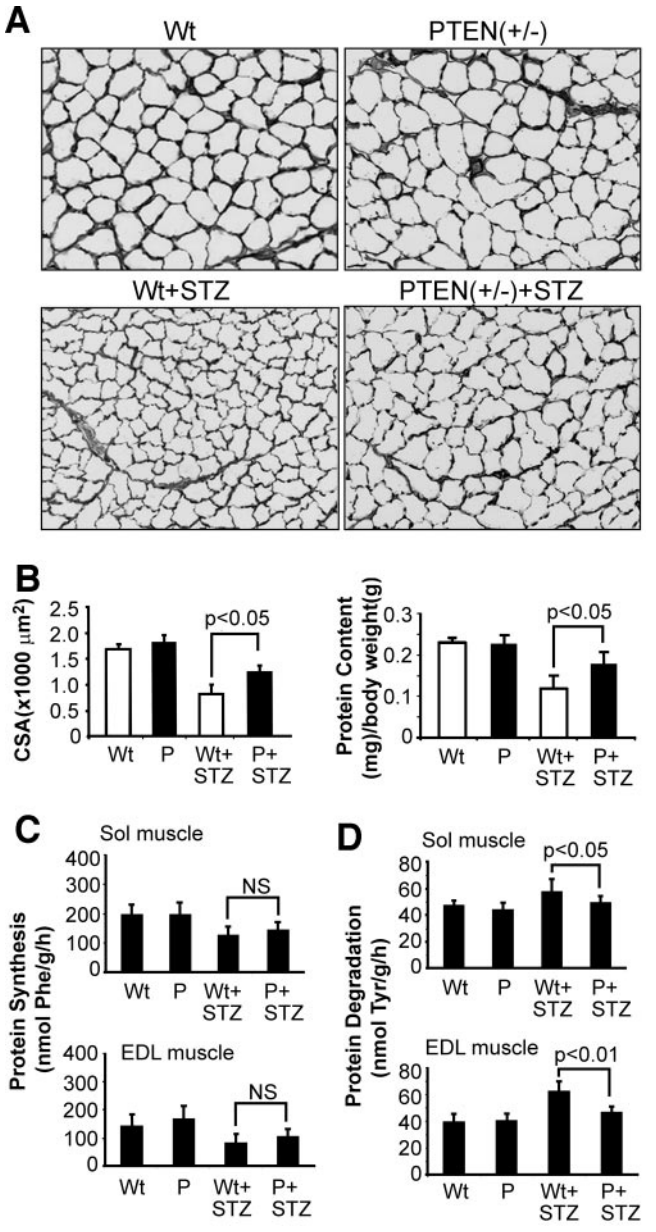

FIG. 4. PTEN regulates muscle protein turnover in vivo. *A*: The cross-sectional areas (CSA) of myofibers in gastrocnemius muscles from wild-type and PTEN$^{+/-}$ mice with or without STZ injection. *B*: The CSA (*left panel*) of gastrocnemius muscle and muscle total protein (*right panel*) of tibialis anterior of PTEN$^{+/-}$ (P) and wild-type (Wt) mice was measured at baseline and after inducing acute insulin deficiency. Bars represent the means ± SE of six mice in each group (n = 6). *C*: Protein synthesis was measured as the rate of incorporation of L-[U-^{14}C]phenylalanine into isolated, incubated soleus (Sol) and extensor digitorum longus (EDL) muscles (24). There was no significant impact of PTEN partial deletion on protein synthesis (n = 8). *D*: Protein degradation was measured as the rate of tyrosine release in isolated and incubated soleus and extensor digitorum longus muscles. Partial deletion of PTEN significantly suppressed the increase in protein degradation induced by acute diabetes (n = 8).

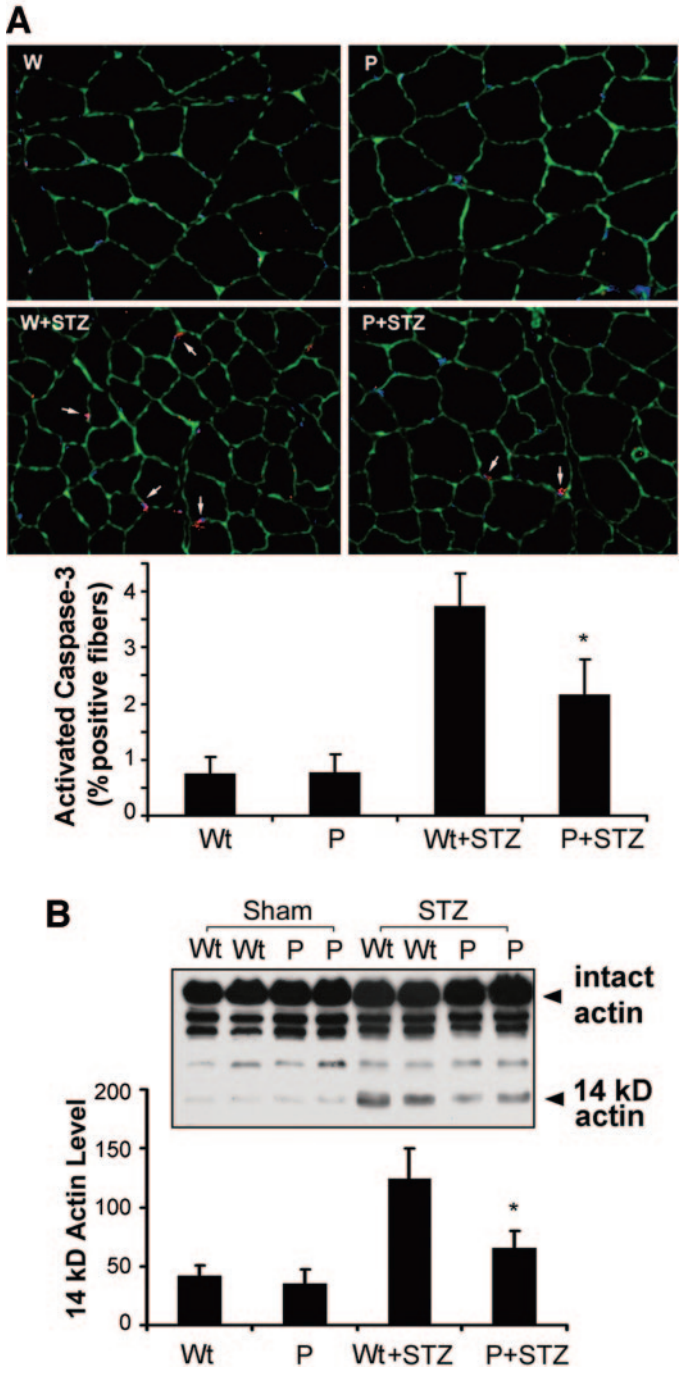

FIG. 5. PTEN influences caspase-3 activity and the changes in actin cleavage. *A*: Activated caspase-3 red immunofluorescence (arrow) in muscles from wild-type (Wt) and PTEN$^{+/-}$ (P) mice with or without STZ treatment; green and blue present laminin and nuclei, respectively. The percentage of muscle fibers containing activated caspase-3 was significantly decreased in muscle of diabetic PTEN$^{+/-}$ mice (*P < 0.05 vs. values in muscle of pair-fed, wild-type mice; n = 6). *B*: Acute diabetes increases the amount of the characteristic 14-kDa actin fragment in muscles, and PTEN$^{+/-}$ attenuated this response (*P < 0.01 vs. values in muscle of pair-fed, wild-type mice; n = 6).

(Fig. 4*C*). In contrast, the rate of protein degradation was significantly suppressed in both types of muscle obtained from diabetic PTEN$^{+/-}$ mice compared with control values from wild-type mice (Fig. 4*D*).

We then examined whether partial deletion of PTEN counteracts two components of muscle proteolysis: actomyosin cleavage mediated by caspase-3 and activation of the UPS. In acute diabetes, activated caspase-3 in individual myofibers was significantly less ($P < 0.05$) in muscle of PTEN$^{+/-}$ mice versus values in pair-fed, wild-type mice (Fig. 5*A*). The resulting characteristic 14-kDa actin fragments were also decreased in muscle of diabetic PTEN$^{+/-}$ mice (Fig. 5*B*) ($P < 0.01$). We evaluated UPS activation by Northern analyses of the E3 ubiquitin–conjugating enzymes atrogin-1/MAFbx and MurF1 mRNAs. Likewise,

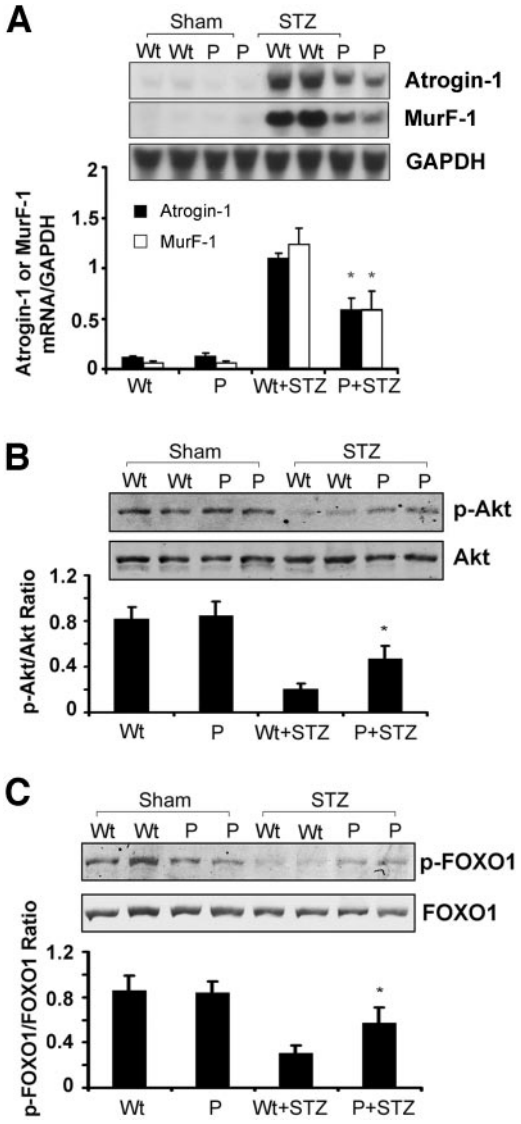

FIG. 6. PTEN partial deletion suppresses atrogin-1/MAFbx and MurF1 mRNAs induced by acute insulin deficiency. *A*: The expression of atrogin-1/MAFbx and MurF1 mRNAs in muscles of mice with acute diabetes and control mice. The increase of those gene expressions was blunted by PTEN partial deletion (*$P < 0.05$ vs. values in pair-fed, wild-type (Wt) mice; $n = 6$). P, PTEN$^{+/-}$ mice. *B* and *C*: PTEN partial deletion increases phosphorylation of Akt and FOXO1 transcription factor in diabetic muscles (*$P < 0.01$ vs. values in pair-fed, wild-type mice; $n = 6$).

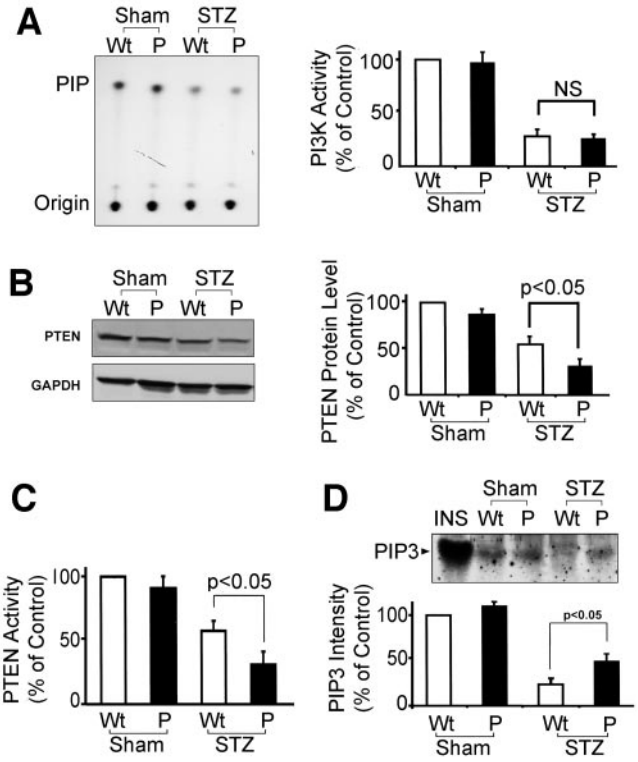

FIG. 7. Partial knockout of PTEN increases the accumulation of PIP_3 in muscle of insulin-deficient mice. *A*: IRS-1–associated PI3K activities in muscles of wild-type (Wt) and PTEN$^{+/-}$ (P) mice are the same at baseline; they also do not differ when there is acute diabetes (*left panel*). The *right panel* contains the statistical analysis of PI3K activity (mean ± SE for four animals in each group; $P < 0.05$). PTEN protein level (*B*) and activity (*C*) were unchanged in untreated mice, but both are decreased in muscle of diabetic PTEN$^{+/-}$ mice (mean ± SE for six animals in each group). *D*: The decrease in PTEN activity in muscle of diabetic PTEN$^{+/-}$ mice caused an accumulation of PIP_3 in muscle (mean ± SE for six animals in each group; $P < 0.05$).

atrogin-1/MAFbx and MurF1 mRNAs were significantly ($P < 0.01$) lower in muscles of acutely diabetic PTEN$^{+/-}$ mice (Fig. 6*A*). Finally, we examined the mechanism that stimulates the expression of atrogin-1/MAFbx by evaluating changes in Akt activity and the activation of FOXO1. We found higher levels of *p*-Akt (Fig. 6*B*) and phosphorylated FOXO1 in muscle of acutely diabetic PTEN$^{+/-}$ mice (Fig. 6*C*). Thus, a decrease in PTEN will suppress two important steps in proteolysis, activation of caspase-3 and the UPS, to provide a mechanism by which decreased PTEN counteracts muscle protein breakdown induced by acute diabetes.

PIP_3 accounts for the antiproteolytic effect of partial PTEN deletion. Acute insulin deficiency decreases IRS-1–associated PI3K activity, but this cannot account for the antiproteolytic influence of PTEN$^{+/-}$ mice because PI3K activities were not statistically different in muscles of the two groups of acutely diabetic mice (Fig. 7*A*). The PTEN protein and the PTEN activity were both significantly lower in muscle of diabetic PTEN$^{+/-}$ mice compared with diabetic pair-fed wild-type mice (Fig. 7*B* and *C*). Consequently, the level of PIP_3 was significantly higher in muscle of diabetic PTEN$^{+/-}$ mice compared with pair-fed diabetic wild-type mice (Fig. 7*D*). This increase in PIP_3 was consistent with an increase in both *p*-Akt and phosphorylated FOXO1 in muscles of acutely diabetic PTEN$^{+/-}$ mice compared with identically treated wild-type mice (Fig. 6*B* and *C*). These results indicate that regulation of muscle proteolysis in acute insulin deficiency results from changes in PTEN as well as PI3K.

DISCUSSION

Recent reports suggest that in many catabolic conditions, decreased signaling through the PI3K/Akt pathways activates muscle protein degradation (3,6,7,29). Contrariwise, when PI3K/Akt signaling is activated, two key proteolytic events are suppressed: actomyosin cleavage and expression of key E3 ubiquitin ligases atrogin-1/MAFbx and MurF1 (7,27,28). In addition, an endogenous mechanism changes the activity of PTEN to regulate changes in muscle protein turnover; a decrease in PTEN yields an increase in PIP_3 and suppression of muscle proteolysis. In muscle of mice with STZ-induced acute diabetes, we found that PTEN is, in fact, downregulated, and in cultured muscle cells when PTEN expression is suppressed, pro-

tein degradation is decreased. Interestingly, the mechanism that suppresses PTEN in cultured myocytes is linked to acute inhibition of PI3K activity.

In contrast to acute diabetes, in chronic diabetes, PTEN mRNA and protein in muscle are increased. This is relevant because an increase in PTEN in chronic diabetes provides an additional positive feed-forward stimulus of muscle protein degradation.

What factors change PTEN expression? PTEN in muscle can be downregulated via a posttranslational modification (30). When we suppressed PI3K by creating acute diabetes or treated C2C12 myotubes with LY294002, PTEN expression, but not PTEN mRNA, decreased (Figs. 1*A* and *B* and 2*C* and *E*). Suggested posttranslational mechanisms that could explain a decrease in PTEN include the study by Torres et al. (31), who found that caspase-3 can cleave the COOH-terminus of PTEN. This is relevant because an acute decrease in insulin activates caspase-3 in muscle (7). Another mechanism affecting the degradation of PTEN is also related to insulin. Birle et al. (32) reported that PTEN is stabilized by PIP_3-induced protein kinase C phosphorylation, suggesting that insulin deficiency could reduce PTEN stability, leading to its degradation.

The mechanism for the increase in PTEN expression with chronic diabetes also appears to be complex, but in this case, the mechanism involves an increase in PTEN mRNA. Wang et al. (26) found that incubating endothelial cells with free fatty acids increases the activity and expression of PTEN. They related the PTEN increase to p38 mitogen-activated protein kinase stimulation. Our findings in cultured C2C12 muscle cells are consistent with these results. We confirmed that palmitic acid increases p38 activation and the expression of PTEN (Fig. 2*A*, *B*, and *D*). In fact, the chronic insulin resistance models that we studied are characterized by higher free fatty acid levels compared with those in animals with acute insulin deficiency (33,34). Finally, we found that inhibition of p38 suppresses the palmitic acid–induced increase in both PTEN mRNA and protein (Fig. 2*A* and *D*). Taken together, the results suggest that PTEN transcription and expression are related to p38 activation.

How do variations in PTEN change muscle protein degradation? The balance between PIP_3 formation and its conversion to phosphatidylinositol 4,5-bisphosphate in muscle changes when PTEN is reduced; PIP_3 levels rise, and protein degradation is suppressed (Figs. 6 and 7). These responses can explain how partial deletion of PTEN counteracts insulin deficiency–induced muscle atrophy even though IRS-1–associated PI3K activity remains low (Fig. 7*A*). The mechanism is due to suppression of actomyosin/myofibril cleavage (Fig. 5*B*) plus an increase in phosphorylated FOXO1, which decreases the expression of atrogin-1/MAFbx and reduces proteolysis in the UPS (Fig. 6). Our finding that the 14-kDa actin fragment was reduced in muscle of acutely diabetic $PTEN^{+/-}$ mice is important because it signifies that an initial step in muscle proteolysis occurred to augment the degree of muscle proteolysis. The same result occurs in several catabolic conditions, including those affecting patients (3,6–8,11, 29). Earlier, we found that there was increased caspase-3 activity and an increase in the 14-kDa actin fragment in muscle of rodents with acute diabetes (7). When we examined the influence of actomyosin cleavage in vitro, there was a large increase in degradation of actomyosin in response to small increases in actomyosin cleavage (6). Likewise, the decrease in atrogin-1/MAFbx in muscle of diabetic $PTEN^{+/-}$ mice is important because Bodine et al. (10) reported that forced expression of atrogin-1/MAFbx in myotubes increases proteolysis and that knockout of the gene suppresses the protein breakdown induced by denervation.

Importantly, we found no difference in the size of muscles of mice with partial PTEN deletion compared with that of littermate wild-type controls unless the mice were challenged by insulin deficiency. These results reflect the equivalent levels of PIP_3 in muscles of nondiabetic $PTEN^{+/-}$ and wild-type mice (Fig. 7*D*). Consistent with these results, Wijesekara et al. (17) reported that *p*-Akt is unchanged in mouse muscle even when there is muscle-specific knockout of PTEN. However, we found that acute insulin deficiency reduces PTEN levels in muscle of both wild-type and $PTEN^{+/-}$ mice, resulting in higher levels of PIP_3 and *p*-Akt in mice with partial PTEN deletion (Figs. 6*B* and 7*D*). Taken together, these results demonstrate that a decrease in PTEN will counteract the muscle proteolysis usually triggered when there is downregulation of PIP_3/*p*-Akt levels (Figs. 4–7).

In contrast to the different mechanisms that change PTEN expression in acute and chronic diabetes, the mechanisms causing muscle proteolysis are similar. A low *p*-Akt leads to activation of caspase-3 and increased expression of atrogin-1/MAFbx; the latter response proceeds from a decrease in *p*-Akt–dependent phosphorylation of FOXOs (7,9,27,28).

How might the responses we identified play a physiologic role in overall metabolism? In normal subjects, short-term fasting could suppress PI3K/Akt signaling and stimulate proteolytic pathways in muscle, producing gluconeogenic amino acids. However, fasting also decreases PTEN to counteract the increase in muscle protein degradation. In this way, protein balance is minimally displaced. In contrast, prolonged insulin deficiency/resistance enhances gluconeogenesis from substrates derived in part from protein degradation in muscle. Consequently, an increase in PTEN acts in concert with a decrease in IRS-1–associated PI3K activity to lower PIP_3. This response would provide a positive forward feed to activate muscle protein breakdown.

In summary, we find that there is variability in the expression and activity of PTEN in different forms of diabetes. This variability exerts physiologically important responses in muscle protein metabolism. Potentially, PTEN could be a therapeutic target for reducing accelerated muscle atrophy.

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