Acidosis impairs insulin receptor substrate-1-associated phosphoinositide 3-kinase signaling in muscle cells: consequences on proteolysis

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Acidosis impairs insulin receptor substrate-1-associated phosphoinositide 3-kinase signaling in muscle cells: consequences on proteolysis. Am J Physiol Renal Physiol 287: F700–F706, 2004. First published May 25, 2004; 10.1152/ajprenal.00440.2003.—Chronic acidosis is a stimulus for proteolysis in muscle in vivo, but the mechanism of this response is unknown. We tested the hypothesis that acidosis or TNF-α, a cytokine whose production increases in acidosis, regulates proteolysis by inhibiting insulin signaling through phosphoinositide 3-kinase (PI3K). In cultured L6 myotubes, acidified (pH 7.1) media did not accelerate the basal protein degradation rate, but it inhibited insulin’s ability to suppress proteolysis. Insulin receptor substrate-1 (IRS-1)-associated PI3K activity was not altered in cells acidified for 10 min but was strongly inhibited in cells incubated at pH 7.1 for 24 h. Phosphorylation of Akt was also suppressed by acidification for 24 h. Acidification did not induce changes in IRS-1 abundance, insulin-stimulated IRS-1 tyrosine phosphorylation, or the amount of PI3K p85 regulatory subunit. In contrast to acidification, TNF-α suppressed proteolysis in the presence or absence of insulin but had no effect on IRS-1-associated PI3K activity. To establish that the PI3K pathway can regulate protein degradation in muscle, we measured proteolysis in cells after inhibition of PI3K activity with LY-294002 or infection with an adenovirus encoding a dominant negative PI3K p85α-subunit. Both approaches inhibited insulin-induced suppression of proteolysis to a degree similar to that seen with acidification. We conclude that acidosis accelerates protein degradation by impairing insulin signaling through PI3K in muscle cells.

of that observed in patients or experimental animals with metabolic acidosis resulting from NH₄Cl ingestion or CKD (12). These proteolytic responses may be related because both conditions activate the same proteolytic pathway, the ubiquitin-proteasome system (1, 36). Furthermore, we found that preventing the spontaneous ketoacidosis of acute diabetes in rats did not block the proteolytic response to insulinopenia (37). Thus the available evidence suggests that acidosis per se does not directly stimulate muscle proteolysis. These findings prompted us to investigate whether acidosis stimulates protein degradation by interfering with intracellular insulin signaling.

Acidosis could stimulate protein catabolism in muscle by several potential mechanisms. Acidosis could produce secondary responses that alter insulin signaling pathways or protein degradation. For example, acidification induces the release of TNF-α from macrophages (2) and infusion of TNF-α into rats has been suggested to stimulate the ubiquitin-proteasome proteolytic pathway in muscle (17, 27). Moreover, inhibition of TNF-α production in animal models of cancer and sepsis can reduce some markers of ubiquitin-proteasome activation in skeletal muscle (4). This is important because muscle can produce TNF-α (24). Acidosis can also increase glucocorticoid production, and elevated glucocorticoids are linked to accelerated proteolysis (31). Because both TNF-α and glucocorticoids have been linked to insulin resistance in skeletal muscle, this raises the possibility that insulin resistance may be a signal for increased protein degradation (9, 21). Another possibility is that extracellular acidification may directly impact insulin signaling or protein degradation without involvement of other secondary mediators.

To isolate the effect of acidification and TNF-α from other systemic factors that could be induced by acidification in an in vivo model of muscle wasting (e.g., glucocorticoids, azotemia), we studied the effect of these signals on protein degradation in the presence and absence of insulin in vitro in L6 rat skeletal myotubes. We found that acidosis, but not TNF-α, blocked the antiproteolytic effect of insulin by attenuating signaling through phosphoinositide 3-kinase (PI3K). We also provide evidence that PI3K directly regulates muscle proteolysis.

MATERIALS AND METHODS

All chemicals or reagents were purchased from Sigma (St. Louis, MO) except DMEM, fetal bovine serum, trypsin-EDTA, and penicillin-streptomycin, which were from GIBCO (Grand Island, NY); six-well cell culture plates were from Corning (Corning, NY); silica
gel thin-layer chromatography plates were from Whatman (Maidstone, Kent, UK); L-[U-14C]phenylalanine (Phe) was from New England Nuclear, DuPont (Boston, MA); anti-p85 PI3K antibodies were from Upstate Biotechnology (Lake Placid, NY); anti-p110 PI3K antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-AKT and phospho-AKT antibodies were from Cell Signal Technology (Beverly, MA); PY-20 anti-phosphotyrosine antibody was from Transduction Laboratories (San Diego, CA); and regular human insulin (Humulin R) was from Eli Lilly (Indianapolis, IN). TNF-α was a generous gift from Amgen (Thousand Oaks, CA).

Cell culture. Rat L6 myoblasts (ATCC, Manassas, VA) at passages 3–7 were grown to confluence in six-well plates containing DMEM with penicillin/streptomycin and 10% FBS in a 95% O2-5% CO2 atmosphere (pH 7.4) (30). Cell differentiation to myotubes was induced by growing myoblasts to 70% confluence and replacing the growth medium with DMEM supplemented with 2% horse serum.

Toxicity of pharmacological inhibitors or adenoviruses was assessed by trypsin blue exclusion as described elsewhere (10). Concentrations of inhibitors or adenoviruses used were usually <25% of the lowest concentration that produces detectable toxicity.

Protein degradation. Protein degradation was measured in differentiated myotubes after the pool of cellular proteins was labeled for 3 days in the presence of 0.5 μCi L-[14C]Phe/well as described elsewhere (3, 46). To study the impact of acidification, HCl was added to the experimental medium to achieve pH 7.1; the pH was confirmed after equilibration in 95% O2-5% CO2. The experimental medium also contained 2% horse serum, 2 mM unlabeled Phe (to minimize reutilization of released L-[14C]Phe) ± 100 nM regular insulin and/or TNF-α (10–50 ng/ml). After the labeling period, all cells were subjected to a 2-h chase in pH 7.4 experimental medium to remove L-[14C]Phe released from short-lived proteins. The chase medium was replaced with 3 ml of fresh experimental medium (either pH 7.4 or 7.1) supplemented with growth factors and enzyme inhibitors as indicated. Serial aliquots of the medium were removed at intervals up to 72 h, and [14C]Phe released from labeled proteins was measured after precipitation of proteins with TCA (10% vol/vol). At the end of the sampling period, cell monolayers were solubilized in 1% SDS (1 ml/well) to determine the radioactivity remaining in the cells, and the rate of protein degradation was determined by calculating the slope of the logarithm of [14C]Phe remaining in cell protein vs. time (11). Apparent protein half-life of the total pool of cellular proteins was calculated as 0.301 divided by the protein degradation rate. Total radioactivity recovered from cells (calculated from the amount released into the media plus that remaining in the cell monolayer) was an indicator of cell viability and did not change with any experimental treatment. Absolute basal rates of proteolysis were somewhat variable. This variability can be ascribed to the use of several different lots of buffer and the variability in the cell number and cell density.

Protein synthesis. Protein synthesis was measured as the rate of incorporation of L-[14C]Phe into acid-insoluble protein in differentiated L6 muscle cells (19). Cells were incubated in experimental media (e.g., pH 7.1 or daily supplements of 100 nM insulin) for 24 h before [14C]Phe was added. Two hours before the measurement, fresh experimental medium supplemented with 0.6 mM unlabeled Phe was added to cells. The assay was initiated by adding experimental labeling medium containing L-[14C]Phe (0.5 μCi/well) plus 0.6 mM unlabeled Phe to ensure equilibration of intracellular and extracellular specific radioactivities. After 4 h, the labeling medium was removed and the cells were rapidly rinsed three times with ice-cold PBS. Proteins were precipitated by adding TCA (10% vol/vol) to the wells, and the plates were incubated on ice for 1 h. The plates were scraped, the precipitated proteins were washed three times with ethanol/ether (1:1), and the remaining precipitate was solubilized in 1 ml of 0.3 M NaOH overnight. Protein content was determined by the Bio-Rad DC method (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Acid-insoluble radioactivity was measured by liquid scintillation counting. Rates of L-[14C]Phe incorporation were normalized for the protein content of each respective well and the specific radioactivity of Phe. Results are expressed as nanomoles of Phe incorporated per milligram protein.

Adenoviral transfection. The adenovirus Ad.Trackp85ΔiSH2 encodes a dominant negative (DN) mutant PI3K p85 subunit with a deletion of the inner SH2 domain (38) plus green fluorescent protein (GFP) (13). The control adenovirus Ad.GFP encodes GFP only and was a generous gift of Dr. B. Vogelstein (Baltimore, MD). These viruses were propagated in HEK-293 cells and purified by gradient density centrifugation in CsCl; final yields were generally 105–106 plaque-forming units. Ad.GFP was used as a transfection control in all experiments involving Ad.Trackp85ΔiSH2.

Confluent L6 cells in DMEM plus 10% fetal bovine serum were incubated for 20 h with Ad.Trackp85ΔiSH2 or Ad.GFP with a multiplicity of infection (MOI) of 25–50 plaque forming units/cell. Fresh medium was added daily for 2 days before cells differentiated in DMEM plus 2% horse serum. Efficiency of transfection was evaluated by fluorescence microscopy. An MOI of 25 typically produced a 50–60% cell transfection efficiency.

PI3K activity. PI3K activity was measured in differentiated L6 cells as described (13, 46). In some instances, cells were incubated with insulin (100 nM) and/or TNF-α (10–50 ng/ml). To study the effects of acidification, the pH of the medium was adjusted to 7.1. On the day of the experiment, cells were washed twice with PBS containing 100 mM NaVO4 and lysed in PBS extraction buffer containing (in mM) 50 HEPES, 137 NaCl, 1 MgCl2, 1 CaCl2, 10 sodium pyrophosphate, 10 NaF, 2 EDTA, 2 Na3VO4, 2 PMSF, and 10 benzamidine as well as 10% glycerol (vol/vol), 1% NP-40 (vol/vol), 10 mg/ml aprotinin, and 10 μg/ml leupeptin. The concentration of extracted proteins was measured using a Bio-Rad DC protein assay. An aliquot of each sample (700 μg protein) was incubated with 4 μg of anti-insulin receptor substrate-1 (IRS-1) antibodies for 2 h. Protein A-Sepharose (60 ml/sample) was added, and the samples were rocked at 4°C overnight. Immunoprecipitates were washed successively in 1) PBS containing 1% NP-40 and 100 μM Na3VO4; 2) 100 μM Tris-HCl (pH 7.5), 500 μM LiCl, and 100 μM Na3VO4; and 3) 100 μM Tris-HCl (pH 7.5), 100 μM NaCl, 1 mM EDTA, and 100 μM Na3VO4. PI3K activity associated with IRS-1 was measured by resuspending the immunoprecipitates in a solution of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 15 mM MgCl2, 100 μM Na3VO4, 20 μM phosphatidylinositol, 1 mM EGTA, and 440 μM ATP (containing [γ-32P]ATP). The reaction mixtures were incubated with gentle agitation at room temperature for 10 min before the

### Table 1. Acidosis attenuates suppression of muscle proteolysis by insulin

<table>
<thead>
<tr>
<th>Protein Degradation Rate, %Control</th>
<th>Control (n = 18)</th>
<th>pH 7.1 (n = 18)</th>
<th>Insulin (n = 17)</th>
<th>Insulin + pH 7.1 (n = 18)</th>
<th>%Inhibition of Insulin Effect by pH 7.1</th>
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<tbody>
<tr>
<td>Protein</td>
<td></td>
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<tr>
<td></td>
<td>100.0±1.4</td>
<td>99.8±1.0</td>
<td>78.0±1.7*</td>
<td>84.1±0.8*†</td>
<td>27.2%</td>
</tr>
<tr>
<td>Half-life, %Control</td>
<td>100.0±1.5</td>
<td>100.0±1.0</td>
<td>128.7±3.1*</td>
<td>118.7±1.1†</td>
<td>34.3%</td>
</tr>
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Values are means ± SE. n. No. of independent measurements. Results from 3 independent experiments were expressed as percentage of the rate measured in control (untreated) cells or the percentage of the calculated apparent half-life of the total protein pool. The rate of total cell protein degradation was measured as described in MATERIALS AND METHODS. *P < 0.05 vs. control treatment. †P < 0.05 vs. insulin treatment.
addition of 20 μl of 8 M HCl and 150 μl of CHCl₃:MeOH (1:1) to stop the reaction. The products were separated by silica gel thin-layer chromatography using a developing solution of CHCl₃:CH₃OH:H₂O:NH₄Cl (60:47:11.6:2). Results were visualized by phosphorimaging or autoradiography. Products comigrating with a phosphatidylinositol phosphate standard were quantified.

Immunoblot analysis. Cells were lysed in PI3K extraction buffer, and proteins were separated by SDS-PAGE. After the proteins were transferred to nitrocellulose membranes, the blots were incubated in blocking buffer consisting of either Tris-buffered saline with 0.1% Tween 20 and 5% powdered milk or 3% BSA. Blots were incubated with primary antibodies overnight, and the blots were developed using an enhanced chemiluminescence system (Amersham).

Statistics. Results are expressed as means ± SE. Differences between two groups were analyzed by Student's t-test, and multiple comparisons were analyzed by analysis of variance using the Student-Newman-Keuls test for post hoc comparisons. Values were considered significant when P < 0.05.

RESULTS

Insulin and protein turnover. We studied the effect of insulin on protein turnover in L6 myotubes because they are a well-characterized model of muscle cells (19). Addition of insulin (100 nM) to the medium once every 24 h over a 72-h period suppressed the basal rate of proteolysis 28 ± 3% (P < 0.05 vs. untreated cells) as determined using a pulse-chase protocol (Fig. 1A). With this method, protein degradation rates are calculated as the slope of the plot of the percentage of [14C]Phe remaining in a cell monolayer vs. time. Cells were also treated with MG132, a proteasome inhibitor, to determine whether insulin inhibits a proteasome-dependent pathway. If so, the suppressive effects of insulin should not be additive to those of MG132. The data in Fig. 1A demonstrate that MG132 eliminated the attenuation of proteolysis by insulin.

Next, we investigated the possibility that acidification could interfere with the ability of insulin to suppress proteolysis. Protein degradation was measured in cells incubated at pH 7.1 or 7.4 ± insulin. Insulin suppressed proteolysis when cells were incubated at pH 7.1 (Fig. 1B), but the suppression was less than in cells incubated at pH 7.4 (P < 0.05, pH 7.4 vs. 7.1 in the presence of insulin). Thus acidification blunts the ability of insulin to suppress proteolysis. To determine whether either

Fig. 1. Acidosis attenuates the insulin-induced suppression of proteolysis. Proteins in L6 cells myotubes were labeled with L-[14C]phenylalanine (Phe) as described in MATERIALS AND METHODS. The rate of protein degradation was calculated as the slope of the plot of the logarithm of total [14C]Phe counts remaining in the cell monolayer after a pulse-chase vs. time. A: cells were treated with vehicle (control) or 0.5 μM proteasome inhibitor MG132 ± 100 nM insulin. MG132 and insulin were added to the cells once every 24 h during the experiment. Results from 1 experiment (n = 6 for each treatment) representative of 3 repeats are shown. B: cells were incubated in control (pH 7.4) or acidic (pH 7.1) medium ± 100 nM insulin; insulin was added once every 24 h during the experiment. Data from a single experiment (n = 6) are expressed as the rate of protein degradation. Data shown are representative of 3 separate experiments. C: results from 1 experiment described in B (n = 6 for each treatment) are plotted as the log counts remaining (y-axis) vs. time. D: cells were treated with no additive (control) or the indicated concentrations of TNF-α; TNF-α was added once every 24 h during the experiment. Results from 1 experiment (n = 6 for each treatment) are shown that are representative of 2 separate experiments. E: cells were treated with vehicle (control) or 10 ng/ml TNF-α ± 100 ng/ml insulin; TNF-α and insulin were added every 24 h during the experiment. Results from 1 experiment (n = 6 for each treatment) are shown that are representative of 3 separate experiments.
insulin or acidification altered the kinetics of the insulin effect, the mean values of each time point for each treatment group were plotted (Fig. 1C). All treatments resulted in first-order kinetics, indicating that neither insulin nor acidification changed the kinetics of overall protein degradation. Moreover, the graph demonstrates that the pulse-chase technique can accurately detect small changes in the proteolytic rates.

An alternate method of expressing proteolytic data is to calculate the mean half-life of the total protein pool using the slope of the protein degradation plots. In a representative experiment, insulin increased the half-life of the protein pool from 49.3 ± 1.6 h in untreated, control cells to 68.3 ± 3.2 h in hormone-treated cells (P < 0.05, n = 6). When cells were incubated in acidified media, the mean protein half-life with insulin was 59.8 ± 0.8 h (P < 0.05 vs. insulin at pH 7.4, n = 6).

During the course of these experiments, we noted some variability in the slopes of the plots between individual experiments. We also noted that different lots of serum and cell passage numbers were used, raising the possibility that these variables were responsible for the interexperimental variation. To address this question, we calculated the protein degradation rate and protein pool half-life of the insulin and acid treatment groups as a percentage of the mean value for untreated, control cells. When the data were expressed in this fashion, the variability was minimized (Table 1). Insulin increased the half-life to 128 ± 3.1% (P < 0.05 vs. control, n = 18) of the rate in control cells; the half-life of the protein pool in acidified, insulin-treated cells was 118 ± 1.1% of the control cell value (P < 0.05 vs. insulin at pH 7.4, n = 17). Although these changes may seem small, similar changes result in significant protein accumulation when sustained over time (10, 11).

We also examined the effect of acidification on protein synthesis. Similar to an earlier study by England et al. (8) in C2C12 muscle cells, neither insulin nor acidification alone or in combination altered the rate of protein synthesis in L6 cells (data not shown). Thus acidification regulates protein turnover in L6 cells primarily by interfering with the ability of insulin to suppress protein degradation.

**TNF-α and protein degradation.** To test whether acidification impairs the ability of insulin to suppress proteolysis by stimulating the autocrine production of TNF-α in L6 cells, we incubated L6 cells with 10, 20, and 50 ng/ml of TNF-α and measured protein degradation. TNF suppressed proteolysis at each concentration of cytokine (Fig. 1D), in contrast to the stimulatory effect TNF-α exerts on proteolysis in vivo (17). The decrease in proteolysis with TNF-α was additive with the suppression by insulin, suggesting that the two signals work by independent mechanisms (Fig. 1E).

**Mechanism of action for acidification.** To investigate how acidification attenuates the insulin-induced suppression of proteolysis, we examined the effect of acute and chronic acidification on signaling through the IRS-1-associated PI3K pathway. PI3K activity was measured in immunoprecipitates of IRS-1 prepared from L6 cell homogenates. Incubation of cells in acidified media for 10 min did not alter basal or insulin-stimulated PI3K activity (data not shown), but there was a marked reduction in maximal PI3K activity (i.e., stimulated by insulin) when cells were incubated at pH 7.1 for 24 h (Fig. 2). TNF-α had no effect on basal or insulin-stimulated IRS-1-associated PI3K activity, a finding consistent with the protein degradation data. Activated PI3K induces several subsequent signaling events, including the phosphorylation (activation) of Akt. Immunoblot analysis of whole cell lysates of L6 cells indicated that Akt remained phosphorylated 24 h after the last addition of insulin in cells incubated at pH 7.4, but acidification blocked the response to insulin (Fig. 3). Thus, compared with control cells, acidification caused a marked reduction in the activity of PI3K and its downstream effectors.

We also investigated whether acidosis affects the signaling process upstream of PI3K. Immunoblot analysis indicates that acidification did not alter either IRS-1 abundance or insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 3). An increase in the level of the p85 regulatory subunit of PI3K has been linked to insulin resistance in L6 cells (16), but acidification did not change the amount of the p85 subunit of PI3K (Fig. 3).

To examine the relationship between PI3K activity and proteolysis in muscle cells, we measured protein degradation in L6 cells incubated with the PI3K inhibitor LY-294002. The inhibitor did not significantly change the rate of basal proteolysis, but it partially blocked the insulin-induced reduction in proteolysis (Fig. 4A). We also measured proteolysis in L6 cells transduced with an adenovirus (Ad.Trackp85ΔiSH2) to express a DN class 1 PI3K p85α protein with its inner SH2 domain deleted. The deletion results in a p85α subunit that binds to the p110 subunit of PI3K without causing activation
Expression of the DN p85α subunit substantially inhibited the insulin-induced phosphorylation of Akt while slightly decreasing total Akt abundance (Fig. 4B). In cells infected with Ad.Trackp85ΔiSH2 and treated with insulin, the ratio of phosphorylated Akt to total Akt infection was decreased by 35 ± 2% compared with the ratio in cells infected with the control adenovirus AdGFP (P < 0.05, n = 3). Expression of the DN p85α subunit also increased the basal rate of proteolysis and attenuated the suppression of proteolysis by insulin (Fig. 4C).

If PI3K regulates a proteasome-mediated pathway, then LY-294002 should not prevent the suppression of protein degradation by insulin in the presence of the proteasome inhibitor MG132. Conversely, if PI3K regulates a different proteolytic pathway (e.g., lysosomal pathways), the effects of MG132 and LY-294002 should be additive. As seen in Fig. 4D, protein degradation was not greater in insulin-treated cells incubated with MG132 plus LY-294002 than with MG132 alone. Thus the data are consistent with the hypothesis that PI3K regulates proteasome-mediated proteolysis in skeletal muscle through a process that may involve an unidentified downstream effector enzyme.

**DISCUSSION**

In studies of protein turnover in humans, the effect of insulin on protein synthesis is controversial, but there is general agreement that it suppresses protein degradation in muscle (28). Consistent with the human studies, Mitch and colleagues (33) reported that acidosis exerts its effect primarily on muscle proteolysis rather than on synthesis in rats. Related studies indicate that acidosis acts indirectly to stimulate protein degradation. In adenorelatedmized rats, acidoses in the absence of glucocorticoids was not sufficient to increase protein catabolism (31). Furthermore, May et al. (33) found that acidosis blunted the antiproteolytic effect of insulin, suggesting that the influence of acidosis on proteolysis is a result of its effect(s) on insulin signaling. Because insulin resistance has been linked to enhanced muscle proteolysis in several clinical conditions including uremia, severe trauma, major surgery, sepsis, burn injury, and cancer (12, 36), we reasoned that acidosis could interfere with the normal suppression of proteolysis by circulating insulin. Our findings are consistent with these studies and this hypothesis.

Under normal physiological conditions, the binding of insulin to its receptor initiates a number of signaling events in muscle cells. One response is the phosphorylation of tyrosine residues in IRS proteins, which facilitates the recruitment of class 1 PI3K, a cytoplasmic enzyme composed of a p85-kDa regulatory subunit and a p110-kDa catalytic subunit (44). We focused our studies on IRS-1 because PI3K activity associated with IRS-1 predominates in skeletal muscle (25). Furthermore, IRS-1 knockout mice exhibit growth retardation even though it is unclear whether glucose transport in skeletal muscle is unperturbed (15, 23). This finding could be indicative of abnormal protein turnover. Our studies in L6 cells indicate that acidification does not affect IRS-1 tyrosine phosphorylation but inhibits the activity of IRS-1-associated PI3K and the phosphorylation of AKT. These findings are similar to defects we identified in IRS-1-associated PI3K signaling in muscle of rats with chronic renal insufficiency complicated by metabolic acidosis (Bailey JL, Zheng B, Price SR, and Mitch WE, unpublished observations). In these rats, IRS-1-associated PI3K activity was suppressed, and correction of their acidosis by feeding them bicarbonate ameliorated the inhibition of PI3K activity.

How does acidification impair insulin signaling through PI3K? While the mechanism remains obscure, our results
suggest that the defect occurs at the level of IRS-1 and PI3K. Others have reported that acidosis does not block insulin binding to its receptor or receptor kinase autophosphorylation activity in muscle of acidic uremic rats or in patients (6, 29, 32). Our results provide additional evidence that the defect is downstream of the insulin receptor because insulin-dependent IRS-1 tyrosine phosphorylation was unaffected by acidification. However, our studies have not excluded other possible mechanisms of insulin resistance. Insulin-induced activation of PI3K is inhibited when several different serine/threonine residues in IRS-1 are phosphorylated (45). In another report, an increased amount of p85 catalytic subunit was associated with decreased PI3K activity (43); however, in our studies, acidification did not change the abundance of p85α.

At first glance, our data on TNF-α seem to contradict a report by Del Aguila et al. (5) that it blocks insulin-stimulated PI3K activity in C2C12 myocytes. However, the responsiveness of human primary myocytes to TNF-α was dependent on their state of differentiation. Treatment of myoblasts with TNF-α reduced insulin-stimulated Akt phosphorylation before fusion but not after fusion and myotube formation. Thus the highly differentiated state of the L6 myotubes in our studies could account for the failure of TNF-α to change PI3K activity. It is notable that TNF-α can evoke some responses in differentiated myotubes because protein degradation was suppressed in its presence (Fig. 1D). However, these findings should not be interpreted to mean that TNF-α does not contribute to the muscle-wasting process. Cytokines, including TNF-α, induce other responses in vivo that could contribute to muscle wasting associated with chronic diseases like CKD. For example, TNF-α inhibits MyoD gene expression and destabilizes MyoD protein, leading to inhibition of myogenic differentiation (20, 26). Such actions would exacerbate muscle wasting by blocking repair pathways that are necessary for myoblast growth and regeneration of muscle mass (42). Thus, even if it does not activate proteolysis in mature myotubes, TNF-α inhibition remains an important target for pharmacological therapies to enhance muscle mass in such conditions as cancer and infection.

Based on our findings, we propose that the IRS-1-associated PI3K pathway regulates muscle proteolysis. A corollary to this hypothesis is that conditions that interfere with signaling through IRS-1-associated PI3K (e.g., acidosis) stimulate protein degradation in muscle. How could a reduction in PI3K activity result in stimulation of protein degradation in muscle cells? For contractile protein complexes to be degraded by the ubiquitin-proteasome system, the complexes must first be dissociated (41) or cleaved into fragments (7). We recently showed that caspase-3 can cleave actin in actomyosin complexes and myofibrils and that inhibition of PI3K increased actin cleavage in L6 muscle cells (7). This process could produce substrates for proteolytic degradation. In two recent reports, IGF-1 and insulin were shown to suppress expression of atrogin-1, a muscle-specific E3 ubiquitin ligase (39, 40). Moreover, a reduction in activity of the PI3K/ Akt pathway was associated with a reduction in C2C12 myotube size and an increase in atrogin-1 expression. Our studies are consistent with these reports because blocking type 1 PI3K with a DN mutant p85 subunit was sufficient to increase the rate of protein degradation in L6 cells. It is interesting that the PI3K pathway does not regulate the same proteolytic systems in all cell types.

In renal cells, growth factors suppress lysosomal proteolysis by a mechanism involving PI3K, but the activity of the ubiquitin-proteasome system is unchanged (11, 13). Thus the regulation of protein catabolism occurs in a cell type-specific fashion. In conclusion, our studies are the first to show that acidification induces defects in insulin signaling that inhibit PI3K activity, resulting in a higher rate of protein degradation in muscle. Our results may also provide insights into the role of insulin resistance in other complications of acidosis.

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