Complications of chronic kidney disease (CKD) include depressed responses to insulin/IGF-1 and accelerated muscle proteolysis as a result of activation of caspase-3 and the ubiquitin-proteasome system. Experimentally, proteolysis in muscle cells occurs when there is suppression of phosphatidylinositol 3-kinase (PI3-K) activity. Postreceptor signaling through the insulin receptor substrate (IRS)/PI3-K/Akt pathway was evaluated in muscles of acidic, CKD and pair-fed control rats under physiologic conditions and in response to a dose of insulin that quickly stimulated the pathway. Basal IRS-1–associated PI3-K activity was suppressed by CKD; IRS-2–associated PI3-K activity was increased. The basal level of activated Akt in CKD muscles was also low, indicating that the higher IRS-2–associated PI3-K activity did not compensate for the reduced IRS-1–associated PI3-K activity. Insulin treatment overcame this abnormality. The low IRS-1–associated PI3-K activity in muscle was not due to a decrease in IRS-1 protein, but there was a higher amount of the PI3-K p85 subunit protein without a concomitant increase in the p110 catalytic subunit, offering a potential explanation for the lower IRS-1–associated PI3-K activity. Eliminating the acidosis of CKD partially corrected the decrease in basal IRS-1–associated PI3-K activity and protein degradation in muscle. It is concluded that in CKD, acidosis and an increase in the PI3-K p85 subunit are mechanisms that contribute to suppression of PI3-K activity in muscle, and this leads to accelerated muscle proteolysis.

Materials and Methods

Induction of CKD

CKD was created in male Sprague-Dawley rats that weighed 50 to 75 g (Charles River Sprague Dawley, Boston, MA), and a pair-feeding regimen was followed as described (1). In some experiments, NaHCO₃ was added to the diet of some rats with CKD to prevent metabolic acidosis (1). Rats were fasted the night before experiments to eliminate the variability in protein metabolism related to differences in food absorption. On the day of the experiment, the rats were anesthetized, muscles were removed, and arterial blood was collected to measure pH and blood gases. All studies were approved by the Emory University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurement of Muscle Protein Degradation

The rate of total protein degradation was measured in mixed-fiber epitrochlearis muscles as described previously (1). Protein turnover rates in this muscle are comparable to those measured in the bulk of muscle in rats (16).

Actin Cleavage Analysis

In muscle, caspase-3 cleaves actin as an initial step in actin degradation (8). Accumulation of a characteristic 14-kDa actin cleavage fragment serves as a useful marker for evaluating protein degradation in skeletal muscle (8,9). The actin peptide was detected in the insoluble fraction of crude lysates of the untreated (i.e., no insulin injection) gastrocnemius muscle. After centrifugation of the lysate, the insoluble fraction was boiled for 20 min in SDS-PAGE loading buffer. The 14-kDa actin fragment was detected by immunoblot analysis using antibodies that detect the carboxy terminus of actin, as described previously (8).

Insulin Infusion

After isolation of the epitrochlearis muscles to measure protein degradation, the hind limb muscles were exposed and a midabdominal incision was made. For studying the IRS/PI3-K/Akt pathway under basal physiologic conditions, the left iliac artery was clamped and the right hind limb muscles were freeze-clamped in liquid nitrogen. This technique allowed us to measure both basal and maximal activity in different experimental animals.

PI3-K Activity

PI3-K activity that was associated with IRS-1 or IRS-2 in muscle was measured in immunocomplexes using methods described by Saltiel and Kahn (18).

Immunoblot Analysis

Muscles were homogenized on ice in 1 ml of freshly prepared extraction buffer (pH 7.4; 50 mM HEPES [pH 7.4], 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM NaF, 2 mM EDTA, 5% glycerol and 1% NP-40, 1 mM NaVO₄, 5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM benzamidine). The homogenate was clarified by low-speed centrifugation, and equal amounts of supernatant proteins were separated by SDS-PAGE. Proteins were transferred to immunoblot membranes and were visualized by Ponceau-S staining. Polyclonal antibodies were used to evaluate signaling proteins: Akt and phospho-Akt (phospho-Ser-473) antibodies were from Cell Signaling Technology (Beverly, MA); PI3-K p85 subunit and IRS-1 and IRS-2 antibodies were from Upstate (Lake Placid, NY). Immunoblots were incubated with the primary antibodies overnight and with horseradish peroxidase–conjugated secondary antibody (1 h) before development using enhanced chemiluminescence.

For measuring the relative amounts of IRS-1 and IRS-2 tyrosine phosphorylation, lysates were prepared and the proteins were immunoprecipitated with a PY20 phosphotyrosine mAb from BD Transduction Laboratories (San Diego, CA). The immunocomplexes were subjected to Western blot analysis with either the anti–IRS-1 or IRS-2 polyclonal antibodies.

Statistical Analyses

Data were expressed as the mean ± SEM. 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. Results were considered statistically significant at P < 0.05.

Results

There were no statistical differences in the weights of the control and CKD rats, but serum urea nitrogen values were strikingly different (19.2 ± 8.7 [control] versus 136.0 ± 5.2 mg/dl [CKD]; P < 0.005). CKD rats had lower serum bicarbonate values than their pair-fed controls (12.0 ± 4.7 [CKD] versus 25.4 ± 3.2 mM [control]; P < 0.01; n = 5 pairs). The arterial blood pH of CKD rats was 7.19 ± 0.17 versus 7.30 ± 0.07 (P < 0.05; n = 5) in the controls. Consistent with our earlier results (1), the rates of protein degradation that we measured in isolated epitrochlearis muscles from CKD rats were higher than the rates that were measured in control rat muscles (Table 1). The increase in muscle proteolysis has been shown to be due principally to activation of the Ub-P’some proteolytic system (1).

After insulin and IGF-1 bind to their receptors, the intrinsic receptor kinase activities phosphorylate tyrosine residues in IRS-1 and IRS-2. These phosphorylated IRS proteins then serve as docking sites to recruit PI3-K. We evaluated the first step in the postreceptor IRS/PI3-K/Akt signaling pathway by assess-

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<th>Table 1. Muscle protein degradation rates in CKD and pair-fed control rats in independent experimentsa</th>
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<td>Protein Degradation Rate (nmol Tyr/h per mg muscle)</td>
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aRatios of total muscle protein were measured in incubated mixed fiber epitrochlearis muscle from chronic kidney disease (CKD) and pair-fed, sham-operated control rats as described in Materials and Methods. Results from two experiments (five rats per treatment group) are reported as mean ± SE.

bP < 0.05 versus control as determined by a paired t test.
ing the relative amounts of IRS-1 and IRS-2 and their activation states in hind limb muscles from CKD and control rats. The level of IRS-1 in muscle was unchanged in CKD rats as compared with control rats, whereas the amount of IRS-2 in muscle was 62% higher (1.48 ± 0.15 versus 0.91 ± 0.06 optical density units [ODU]/mm², respectively; *P* < 0.05; *n* = 5 pairs) in CKD rats (Figure 1A). Ponceau-S staining of the protein blots indicated that protein loading was equal, confirming that IRS-2 levels were higher (Figure 1B). Tyrosine phosphorylation of IRS-1 and IRS-2 was difficult to detect under the basal physiological condition (*i.e.*, with *in vivo* levels of insulin and IGF-1; Figure 1C). After injection of a pharmacologic dose of insulin, the amount of IRS-1 that was phosphorylated on tyrosine residues in *in vivo* was variable, and there was no consistent pattern of increase or decrease. In contrast, IRS-2 phosphorylation was consistently increased an average of 238% (0.95 ± 0.22 [CKD] versus 0.40 ± 0.10 ODU/mm² [controls]; *P* < 0.05; *n* = 5 pairs; Figure 1C). In response to insulin, the ratio of phosphorylated:total IRS-1 in CKD muscle was not statistically different from controls. Calculation of the same ratio for IRS-2 revealed that IRS-2 tyrosine phosphorylation in muscle was increased 67% by CKD (Figure 1C).

Class 1A PI3-K is a heterodimeric subunit complex that is composed of regulatory and catalytic subunits, and its activity is determined, in part, by the relative amounts of each component (19). Therefore, we examined the levels of each subunit type in muscle. The amount of p85 regulatory subunit in CKD was 79% higher than that in controls (0.70 ± 0.10 versus 0.39 ± 0.05 ODU/mm², respectively; *P* < 0.05; *n* = 5 pairs); the p110 catalytic subunit level was not different (Figure 1D). To determine whether the increase in p85 was associated with altered PI3-K function, we examined the basal and maximal PI3-K activities that were associated with IRS-1 or IRS-2 (Figure 2). With *in vivo* levels of insulin, IRS-1–associated PI3-K activity was suppressed 53% in muscles of CKD rats versus pair-fed control rats (0.32 ± 0.09 [CKD] versus 0.67 ± 0.14 ODU/mm² [controls]; *n* = 5 pairs). After insulin injection, the CKD-related defect in IRS-1–associated PI3-K activity was eliminated (3.75 ± 0.38 [CKD] versus 3.65 ± 0.22 ODU/mm² [controls]; *n* = 5 pairs; Figure 2). In the case of IRS-2–associated PI3-K, it was surprising that both the basal activity (0.23 ± 0.10 [CKD] versus 0.09 ± 0.04 ODU/mm² [controls]; *n* = 5 pairs; *P* < 0.05) and the insulin-stimulated activity (2.61 ± 0.41 [CKD] versus 0.86 ± 0.18 ODU/mm² [controls]; *n* = 5 pairs; *P* < 0.05) were increased 255 and 303%, respectively, in muscle.

Because CKD induces reciprocal responses in basal IRS-1– and IRS-2–associated PI3-K activities, it was unclear how the critical downstream signaling effector Akt would change. Therefore, we analyzed the total and phosphorylated Akt (phospho-Ser473) levels in muscles of CKD and control rats. Under basal conditions, phosphorylated Akt was 39% lower in CKD muscles compared with that in controls (0.32 ± 0.09 versus 0.50 ± 0.08 ODU/mm², respectively; *P* < 0.05; *n* = 5 pairs; *P* < 0.05) and the insulin-stimulated activity (1.35 ± 0.21 [CKD] versus 3.73 ± 0.78 ODU/mm² [controls]; *n* = 5 pairs; *P* < 0.05) was increased 183 and 200%, respectively, in muscle.

**Figure 1.** Insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2 proteins in muscle of rats with chronic kidney disease (CKD). (A) Immunoblots of IRS-1 and IRS-2 proteins in gastrocnemius muscle of two pairs of control rats (CTL) and CKD rats. (B) The blot from A stained with Ponceau-S to visualize the transferred proteins before immunoanalysis. (C) The left gastrocnemius muscle of CTL or CKD rats was untreated before harvesting, whereas the right leg muscles were infused with insulin for 2 min *in vivo* as described in Materials and Methods. The muscles were harvested, IRS-1 and IRS-2 were immunoprecipitated with isoform-specific antibodies, and immunoblot analysis was performed using a mAb (PY20) that detects phosphotyrosine residues. Results from two sets of CKD and paired CTL rats are shown. (D) Amounts of phosphatidylinositol 3-kinase (PI3-K) enzyme p85α regulatory and p110α catalytic subunits in gastrocnemius muscle of CTL and CKD rats were compared by immunoblot analysis. Results from two pairs of CKD and CTL rats are shown.

**Figure 2.** PI3-K activity associated with IRS-1 and IRS-2. The left gastrocnemius muscle of CTL or CKD rats was untreated before harvesting, whereas the right leg muscles were infused with insulin for 2 min *in vivo* as described in Materials and Methods. IRS-1 and IRS-2 were immunoprecipitated from gastrocnemius muscle lysates with isoform-specific antibodies, and PI3-K activity was measured. Reaction products were separated by thin-layer chromatography; the origin is at the bottom, whereas the reaction product, phosphatidyl inositol phosphate, migrates near the top. Duplicate reactions were performed from each lysate, and results from both assays are shown side by side.
Figure 3. This decrease was not due to a reduction in the cellular content of Akt (Figure 3). When the pathway was maximally stimulated with insulin, the amount of phosphorylated Akt in muscle of CKD was not different from that in muscles of pair-fed control rats (3.01 ± 0.26 versus 2.53 ± 0.14 ODU/mm², respectively; NS; n = 5; Figure 3). Because the IRS-1–associated PI3-K activity in vivo is reduced, these results indicate that basal signaling through the PI3-K/Akt pathway is suppressed by CKD. Moreover, a high dose of insulin can overcome the influence of a low IRS-1–associated PI3-K activity in muscle of CKD rats.

There is evidence that metabolic acidosis induces insulin resistance in otherwise normal adults (20), and in earlier studies, we demonstrated that acidosis accelerates muscle proteolysis in rats with CKD (1). Others reported that whole-body protein degradation is increased in acidicotic patients with CKD (21–23). To determine the contribution of metabolic acidosis to the signaling defects of IRS-1/PI3-K, we studied CKD rats that were fed NaHCO₃ to correct their spontaneous acidosis (1). In this experiment, CKD rats had an arterial blood pH of 7.15 ± 0.06 (P < 0.05 versus other groups; n = 5/group) versus 7.43 ± 0.05 for CKD-bicarbonate rats and 7.37 ± 0.04 for control rats. We evaluated protein degradation as the level of the 14-kD actin fragment accumulated in the insoluble fraction of a muscle homogenate because we have demonstrated that CKD increases caspase-3–mediated cleavage of actin in muscle and leaves a characteristic 14-kD peptide fragment that is detectable with an antibody to the C-terminus of actin (8,9). Consistent with our earlier results, the actin fragment was more abundant in gastrocnemius muscle of CKD rats than in control muscle (51.6 ± 9.7 versus 18.2 ± 5.3 ODU/mm², respectively; P < 0.05 versus controls; n = 5/group; Figure 4). The amount of fragment detected in muscle of CKD-bicarbonate rats (30.4 ± 8.3 ODU/mm²; n = 5) was lower than in CKD rats but was greater than in controls.

Basal activity of IRS-1–associated PI3-K activity in muscle of rats that were fed bicarbonate was intermediate (0.62 ± 0.17 ODU/mm²) compared with the activities measured in muscle extracts from CKD and control rats (0.23 ± 0.02 and 0.92 ± 0.23 ODU/mm², respectively; Figure 5); both latter groups were significantly different (P < 0.05; n = 5/group) from the values of the CKD-bicarbonate group or each other. After insulin treatment, there was no statistical difference in the activities measured in the three groups (4.05 ± 1.31[CKD], 5.13 ± 1.07 [CKD-bicarbonate], and 5.93 ± 1.09 ODU/mm² [controls]; n = 5/group).

Discussion

In a review, Smith and Defronzo (24) suggested that Neubauer’s first description of abnormal glucose metabolism among uremic patients was the genesis for investigating this abnormality. Besides defects in glucose utilization (25), there is evidence that insulin resistance is related to defects in muscle protein turnover. May et al. (26) reported that several aspects of insulin-mediated glucose and protein metabolism were abnormal in muscle of acutely uremic rats. The metabolic abnormalities were attributed to unidentified postinsulin defects; the influence of metabolic acidosis was not evaluated.

Others have examined the characteristics of partially purified insulin and IGF-1 receptors from muscle of uremic rodents (acute or chronic) with inconsistent results (6,14,15). Identifying cell signaling abnormalities in these types of experiments is complicated because in vitro measurements of insulin and IGF-1 receptor functions may not reflect accurately the physiologic characteristics of the receptors in vivo. To our knowledge, our experiments are the first to identify in vivo a set of abnor-
nal, postreceptor signaling events in the insulin/IGF-1 pathway. Specifically, we found functional abnormalities in the IRS/PI3-K cascade that reduce the phosphorylation (i.e., activation) of the critical downstream effector Akt (protein kinase B) in muscle of rats with CKD. It is interesting that these abnormalities were overcome when the signaling pathway was maximally stimulated with insulin. We could not determine whether this response to insulin is related solely to its receptor, because the high dose of insulin could have activated both the insulin and IGF-1 receptors. Because both receptors transduce into common signaling pathways, the low basal levels of IRS-1–associated PI3-K activity and phosphorylated Akt indicate that there are physiologic abnormalities associated with both insulin and IGF-1 signaling in vivo. This is important because reductions in these signaling events have been shown to stimulate protein degradation in muscle (8,9,27–30).

In these studies, we cannot exclude the possibility that the low PI3-K activity in muscle was secondary to low concentrations of insulin and/or IGF-1. We did not assess insulin resist ance by the classic insulin clamp technique or measure plasma and muscle levels of IGF-1 and its binding proteins, because it was not possible to perform these analyses and evaluate the in vivo, physiologic changes in PI3-K activities and protein degradation in muscle of the same animals. It seems unlikely, however, that low levels of insulin/IGF-1 are the sole cause of the signaling abnormalities that we identified. Uremia and acidosis typically have been associated with normal or high insulin levels (2,14,26,31–34). Moreover, it is unclear how low values of insulin/IGF-1 would raise the muscle levels of the IRS-2 and PI3-K p85 subunit or increase basal IRS-2–associated PI3-K activity. Ding et al. (6) reported that plasma and muscle levels of IGF-1 are subnormal in muscle of CKD rats, but they also found that IGF-1 failed to attenuate the excessive muscle protein degradation in these rats. Why the resistance to the anabolic effects of IGF-1 was associated with a low IGF-1 level is not clear. The findings, however, support the common theme that CKD induces resistance to the metabolic effects of insulin/IGF-1.

Initially, we were surprised to find that CKD selectively increased IRS-2. Although the IRS proteins have similar amino acid sequences and secondary structure domains, they are differentially expressed in various tissues, and distinct functions have been ascribed to the different isoforms (18). For example, the functions of IRS-1 and IRS-2 were reported to be nonredundant in L6 myotubes (35); IRS-1 was responsible for insulin-stimulated Akt1 phosphorylation, actin remodeling, GLUT4 translocation, and glucose uptake, whereas IRS-2 selectively regulated extracellular signal–regulated kinase signaling. In skeletal muscle of normal animals, IRS-1–associated PI3-K activity predominates and IRS-1 knockout mice exhibit glucose intolerance, peripheral resistance to insulin (decreased glucose uptake in adipocytes), and retarded somatic growth (36). The abnormalities in growth and muscle metabolism in IRS-1−/− mice are not secondary to abnormal glucose metabolism (36) and hint that the gene deficiency causes abnormal muscle protein metabolism. Our finding of a low IRS-1–associated PI3-K activity is consistent with this hypothesis. In contrast, mice null for IRS-2 (IRS-2−/−) exhibit insulin resistance in both liver and peripheral tissues; pancreatic β cell mass is reduced, leading to diabetes with impaired growth (37). It is notable that IRS-2 expression is increased by CKD (Figure 1) as it is in muscles of IRS-1−/− mice (38), suggesting that there is a secondary compensation to offset the decrease in basal IRS-1 signaling. However, this increase in IRS-2 protein content in CKD rat muscle did not overcome the reduced basal level of Akt phosphorylation. These findings are relevant to the mechanism that causes accelerated muscle protein degradation in CKD because Akt regulates proteolysis (9,27–29).

What mechanism(s) could account for the depressed PI3-K signaling in muscle of CKD rats? The mammalian PI3-K complex is a heterodimer that is composed of 85-kd (p85) regulatory and 110-kd (p110) catalytic subunits. The p85 regulatory subunit forms a stable, high-affinity complex with the p110 catalytic component; however, the amounts of these subunits in rat muscle are differentially regulated by stimuli such as glucocorticoids (17). Giorgino et al. (39) reported that treating L6 skeletal muscle cells with dexamethasone markedly increased the expression of the p85 protein but only modestly raised the p110 cellular content. These responses were linked significantly to a reduced IRS-1–associated PI3-K activity, and it was proposed that competition between the free p85 subunit and the PI3-K enzyme complex at the binding site on IRS-1 caused the decrease in PI3-K activity. Others reported that glucocorticoids decrease IRS-1–associated PI3-K activity in rat skeletal muscles (17,40). In earlier studies, May et al. (7) reported that glucocorticoid production is increased by CKD, and we found that both metabolic acidosis and acute insulin deficiency require glucocorticoids for stimulation of the Ub-P’some system (11,41). Importantly, May et al. (7) also found that even after correction of acidosis, CKD is associated with a significant increase in glucocorticoid production above the level measured in control rats. This would be consistent with our finding that correction of the acidosis of CKD did not fully restore the reduced IRS-1–associated PI3-K activity in muscle to the level measured in control rats. We conclude that glucocorticoids could contribute to the defective PI3-K activity in CKD rat muscle by increasing the amount of the PI3-K p85 subunit but not the p110 protein.

Glucocorticoids are not the only signal that could regulate muscle PI3-K activity in CKD. We found that acidification of L6 muscle cells (i.e., myotubes) reduced PI3-K activity associated with IRS-1 and blunted the ability of insulin to suppress protein degradation (13). In the same study, TNF-α did not change IRS-1–associated PI3-K activity in L6 myotubes even though it has been proposed to induce insulin resistance and stimulate muscle proteolysis (42–44). Reaich et al. (45) reported that hyperinsulinemia decreased protein degradation before and after the correction of metabolic acidosis. This finding in patients is consistent with our results that show that a pharmacologic dose of insulin will overcome the defect in IRS-1/PI3-K activity in muscle of rats with CKD.

How does a reduction in IRS-1–associated PI3-K activity act as a stimulus for protein degradation? PI3-K, acting through phosphorylated Akt, suppresses the activity of caspase-3 and the FOXO1 and FOXO3 transcription factors (9,46,47). A de-
crease in activated Akt not only stimulates caspase-3 but also increases the transcription of atrogin-1/MAFBx and MuRF1, two muscle-specific E3 ligases that are linked to muscle atrophy in CKD and other catabolic states (9,46–48). Exposure of myotubes to IGF-1 and insulin inhibits caspase-3 activity (and actin degradation) by a mechanism that involves PI3-K (8,9,49). Because IRS-1 has been reported to be the predominant IRS protein involved in the antipapoptotic effects of IGF-1 (50), it could explain why feeding bicarbonate to CKD rats resulted in a decrease in the amount of 14-kD actin fragment in muscle and an increase in IRS-1–associated PI3-K activity in comparison with findings in muscle of acidic CKD rats.

Conclusion
We have identified abnormalities in the insulin/IGF-1 signaling pathway in muscles of CKD rats: IRS-1–associated PI3-K activity and phosphor-Akt are suppressed, but IRS-2–associated PI3-K activity is increased. A potential mechanism for the reduction in IRS-1–associated PI3-K activity is induction of the p85 PI3-K regulatory subunit, but other mechanisms may be involved. Because a low PI3-K activity stimulates muscle protein degradation by activating caspase-3 and the Ub-Psome pathway (8,13), our results provide a biochemical explanation for the accelerated muscle protein degradation that is associated with CKD.

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