Leucine-stimulated mTOR signaling is partly attenuated in skeletal muscle of chronically uremic rats

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Chen Y, Sood S, McIntire K, Roth R, Rabkin R. Leucine-stimulated mTOR signaling is partly attenuated in skeletal muscle of chronically uremic rats. Am J Physiol Endocrinol Metab 301: E873–E881, 2011. First published July 26, 2011; doi:10.1152/ajpendo.00068.2011.—The branched-chain amino acid leucine stimulates muscle protein synthesis in part by directly activating the mTOR signaling pathway. Furthermore, leucine, if given in conjunction with resistance exercise, enhances the exercise-induced mTOR signaling and protein synthesis. Here we tested whether leucine can activate the mTOR anabolic signaling pathway in uremia and whether it can enhance work overload (WO)-induced signaling through this pathway. Chronic kidney disease (CKD) and control rats were studied after 7 days of surgically induced unilateral plantaris muscle WO and a single leucine or saline load. In the basal state, 4E-BP1 phosphorylation was modestly depressed in non-WO muscle of CKD rats, whereas rpS6 phosphorylation was nearly completely suppressed. After oral leucine mTOR, S6K1 and rpS6 phosphorylation increased similarly in both groups, whereas the phospho–4E-BP1 response was modestly attenuated in CKD. WO alone activated the mTOR signaling pathway in control and CKD rats. In WO CKD, muscle leucine augmented mTOR and 4E-BP1 phosphorylation, but its effect on S6K1 phosphorylation was attenuated. Taken together, this study has established that the chronic uremic state impairs basal signaling through the mTOR anabolic pathway, an abnormality that may contribute to muscle wasting. However, despite this abnormality, leucine can stimulate this signaling pathway in CKD, although its effectiveness is partially attenuated, including in skeletal muscle undergoing sustained WO. Thus, although there is some resistance to leucine in CKD, the data suggest a potential role for leucine-rich supplements in the management of uremic muscle wasting.

Chronic kidney disease; branched-chain amino acids; mammalian target of rapamycin signaling pathway; skeletal muscle overload

ABNORMALITIES IN PLASMA and skeletal muscle amino acid (AA) levels are present in advanced chronic kidney disease (CKD) and contribute to the loss of muscle mass common in these patients (8, 23). Often progressive, skeletal muscle wasting causes a loss of strength and function and leads to an increase in morbidity and mortality (27, 30, 38). Uremic muscle wasting, reflecting altered protein degradation and synthesis (36, 46), arises from multiple causes, including reduced food intake, altered neuropeptide signaling, acidosis, inflammation, diabetes, and acquired resistance to or deficiency of anabolic hormones (18), and is worsened by reduced physical activity as CKD advances (30). In general, in patients with advanced CKD, plasma levels of nonessential AAs are elevated, whereas several essential AAs (EAA), including branched-chain AAs (BCAA) such as leucine, are reduced below normal (8, 23). In muscle, valine concentrations are low (5). These abnormalities arise largely because of impaired dietary intake, urinary protein losses, removal of AAs during dialysis, reduced renal metabolism and synthesis, and increased BCAA catabolism in muscle when acidosis supervenes (18, 44). Given this, the standard of care for advanced CKD patients includes provision of an adequate protein/amino acid intake, and clinical trials examining the long-term benefits of nutritional supplements are in progress (18, 20, 29).

EAAs in nutrient supplements serve as substrates for new protein synthesis, stimulate insulin release, and, largely independent of insulin, directly activate the nutrient-sensitive mammalian target of rapamycin (mTOR) signaling pathway (13, 52), which promotes protein synthesis, cell growth, and metabolism (3, 16, 32). BCAAs, primarily leucine, are the most effective AAs for stimulating protein synthesis through the mTOR pathway (32). This process, which is incompletely understood, begins with cell surface AA transporters (28), including the pH-sensitive sodium-coupled neutral AA transporter SNAT2 (19), that sense extracellular AA availability and regulate free intracellular AA levels. Once inside the cell, it is hypothesized that AAs stimulate mTORC1 through the class III phosphatidylinositol 3-kinase (PI3K), Vps34 (25, 42), mTOR exists in two structurally and functionally distinct multiprotein complexes, mTORC1 and mTORC2 (14). mTORC2 is concerned largely with the regulation of the cytoskeleton and cell survival, whereas mTORC1, a nutrient-sensitive regulator, plays an key role in regulating protein synthesis and cell size. mTORC1, henceforth referred to as mTOR, functions by phosphorylating and thus activating multiple downstream signaling proteins. One important mTORC1 target is the eukaryotic translation initiation factor (eIF)-4E-binding protein 1 (4E-BP1), a translational repressor. 4E-BP1 suppresses mRNA cap-dependent translation by binding to the eIF4E translation initiation factor; phosphorylation of 4E-BP1 inhibits this interaction and thus leads to activation of mRNA translation (32, 35). Another mTOR target is the serine/threonine protein kinase p70 S6 kinase 1 (S6K1), which after being activated phosphorylates several proteins, including the 40S ribosomal protein S6 (rpS6), and these steps lead to an increase in protein synthesis (35, 57).

mTOR is also activated by growth factors such as insulin and IGF-I but in contrast to AAs does so via the PI3K/Akt pathway (35). It turns out that, together with the BCAA, insulin coordinately regulates mTOR signaling and protein synthesis (2, 13). Mechanical stretch of muscle is another activator of mTOR and does so through the PI3K/Akt and MAPK pathways (16, 51), and this leads to an increase in protein synthesis and eventually muscle mass. In CKD rats, activation of the

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PI3K/Akt/mTOR pathway by insulin-IGF-I is attenuated (4), but fortunately, the response to mechanical work appears to be maintained (10, 55).

Several studies in advanced CKD patients, including those requiring dialysis, have shown that regular exercise, including resistance, endurance, or combined modalities, can counteract the loss of muscle mass and function common in these patients (30, 38). Apparently, even a short bout of exercise can augment the anabolic response to nutrient supplements given during hemodialysis (37, 45), and in healthy young and old subjects the anabolic response to exercise can be enhanced by EAA supplements (17, 47). Especially effective in normals are leucine-rich supplements, and when combined with resistance exercise they increase mTOR signaling and muscle protein synthesis to a level greater than exercise alone (15, 17).

Although it is well established that uremia impairs the signaling response to anabolic factors such as growth hormone, insulin, and IGF-I (4, 50), the impact of uremia on leucine-stimulated mTOR anabolic signaling in skeletal muscle is not known. In this regard it has been reported that leucine-stimulated insulin secretion is impaired in pancreatic islets isolated from CKD rats (43). Furthermore, leucine resistance with impaired anabolic signaling has been described in sepsis, endotoxemia, aging, and excessive alcohol or glucocorticoid exposure and is due largely to impaired signaling via the mTOR pathway (21, 31, 34, 35, 48). Thus the first aim of this study carried out in rats was to determine whether leucine can effectively stimulate the mTOR anabolic signaling pathway in the presence of CKD. Our second aim was to test whether in CKD leucine can enhance the anabolic signaling seen in response to increased skeletal muscle workload (11).

MATERIALS AND METHODS

Experimental animals and protocols. CKD was induced in male Sprague-Dawley rats weighing 140 ± 1 g (Harlan Laboratories, Madison, WI) by a 5/6 nephrectomy exactly as described before (11). Control animals weighing 139 ± 2 g underwent a sham nephrectomy (SN) operation, and these animals were then given a daily allowance of standard laboratory rat food equal to the average amount of food consumed by the CKD rats the previous day. Twenty-one days later, CKD and SN rats underwent surgery to produce unilateral work overload (WO) of the plantaris muscle. As before (11), under ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia and through a skin incision, the distal two-thirds of the gastrocnemius muscle and its tendon were excised. No surgery was performed on the contralateral limb, which served as a paired nonloaded control. After 7 days of WO, the rats (now 4 wk post-5/6 nephrectomy or SN) were fasted overnight and gavaged the following morning with leucine (Leu) (1.35 g/kg body wt) in water or an equal volume of saline (Sal). Four groups of rats were studied: group 1, CKD-Sal; group 2, SN-Sal; group 3, CKD-Leu; group 4, SN-Leu. The quantity of leucine gavaged approximated that consumed daily by rats (34). Thirty minutes following saline or leucine administration, rats were euthanized and plantaris muscles from both hindlimbs collected, weighed, and frozen at −80°C. Blood was collected, and plasma creatinine, blood urea nitrogen, and CO2 levels were measured with a Beckman LX 20 Analyzer (Beckman Coulter, Fullerton, CA). Ten minutes before euthanization, to measure protein synthesis (24), a bolus of L-[1-14C]phenylalanine (150 mM, 30 μCi/ml, 1 ml/100 g body wt) was given, but because of methodological concerns the data are not presented. Terminal plasma insulin and, for technical reasons, total BCAA were measured with commercial enzyme-linked immunosorbent assay kits from Millipore (St. Charles, MO) and BioVision Research Products (Mountain View, CA), respectively. Plasma leucine levels in tail vein blood collected 3 days prior to euthanization were measured by a nonderivatized, ion-paired, reverse-phase chromatographic method on an Agilent Model 6460 MS/MS by the Biochemical Genetics Laboratory, Stanford University. Animal protocols were approved by the local Veterans Administration Institutional Animal Care and Use Committee.

Western blotting. Antibodies that detect mTOR, phospho-mTOR (Ser2448), 4E-BP1, phospho-4E-BP1 (Thr70), S6K1, phospho-S6K1 (Thr389), rpS6, phospho-rpS6 (Ser235/236), and phospho-Akt (Thr308) were from Cell Signaling Technology (Danvers, MA). Akt and STAT5 (detects STAT5a and -b) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and GAPDH was from Abcam (Cambridge, MA). As before (10, 50), ~70 mg of frozen tissue was homogenized in Triton buffer (100 mM Tris, pH 7.4, 1% Triton X-100) containing 100 mM sodium pyrophosphate, 100 mM NaF, 10 mM EDTA, 10 mM Na3VO4, 2 mM PMSF, and 0.1 mg/ml aprotonin. The samples were electrophoresed through an SDS-polyacrylamide gel, electroblotted onto nitrocellulose membranes, immunodetected with appropriate antibodies, and visualized by enhanced chemiluminescence with exposure to Kodak XAR film (Eastman Kodak). Phosphoproteins were immunodetected first, followed by the specific total protein, after the blot was stripped. For enhanced visualization and analysis of 4E-BP1, transferred proteins were fixed to the membrane by 0.05% glutaraldehyde in TBS-0.02% Tween for 15 min at room temperature (55). In some instances the blots were stained with the Ponceau S stain (Sigma-Aldrich, St. Louis, MO). Phosphoprotein and protein expression were scanned and then quantitated with ImageJ software (National Institutes of Health).

Data analysis and statistics. In the first part of this study directed at examining leucine-stimulated mTOR signaling in nonloaded muscle, signaling protein levels were corrected by the STAT5 protein level that was measured after the Western blot was stripped. STAT5 was selected to correct for loading and transfer errors because STAT5 is unaffected by CKD (50). In the second part of this study examining signaling in WO muscle, we used GAPDH or a Ponceau S-stained ~150-KDa protein (mTOR blot only) to correct for loading (7) instead of STAT5, because the latter increased in WO muscle. To control for variability between blots, an internal control sample prepared from SN pools and run in duplicate was included in the assays, and the results normalized to this sample. Relative phosphorylated protein levels were calculated by normalizing for the respective total protein. The control SN-Sal group mean was given a value of 100, and individual values were expressed relative to this value. Results are given as means ± SE. Two-tailed paired, when comparing WO and non-WO muscle from same animal, and unpaired Student’s t-tests were used for comparison of two normally distributed groups. Comparisons between more than two normally distributed groups were made by a one-way ANOVA, followed by pairwise multiple comparison with the Duncan test. For nonnormally distributed groups, comparisons were made with Dunnett T3 test. The SPSS 17.0 statistical program was used. A P value of <0.05 was considered significant.

RESULTS

Renal function and anthropometrics. After 28 days of CKD, plasma creatinine and urea nitrogen levels were approximately threefold higher than the SN values (Table 1). In contrast, plasma bicarbonate levels did not differ between the two groups. Final body weight was reduced in CKD, and over the 28-day period of comparable food intake the CKD rats gained significantly less weight than the SN controls. Unlike our previous CKD studies in which muscle atrophy was evident (11, 53), nonloaded plantaris muscle weight did not differ between the CKD and SN groups. Similarly, anterior tibial...
Table 1. Biochemical and anthropometric characteristics of SN and CKD rats

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<th>SN</th>
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<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.28 ± 0.01</td>
<td>0.90 ± 0.03**</td>
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<tr>
<td>Plasma urea nitrogen, mg/dl</td>
<td>13 ± 1</td>
<td>47 ± 2**</td>
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<tr>
<td>Plasma bicarbonate, mmol/l</td>
<td>24 ± 0.5</td>
<td>25 ± 1.0</td>
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<td>Body weight gain, g</td>
<td>107 ± 4</td>
<td>92 ± 4*</td>
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<tr>
<td>Final body weight, g</td>
<td>296 ± 4</td>
<td>274 ± 5**</td>
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<tr>
<td>Nonloaded plantaris weight, mg</td>
<td>335 ± 11</td>
<td>354 ± 10</td>
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<tr>
<td>Overloaded plantaris weight, mg</td>
<td>554 ± 22#</td>
<td>547 ± 26#</td>
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Values are means ± SE; n = 12 rats. SN, sham nephrectomized; CKD, chronic kidney disease. Body weight gain was measured over 28 days of comparable food intake postsurgery. *P < 0.05, **P < 0.01, CKD vs. SN; #P < 0.01, overloaded vs. nonloaded muscle.

(600 ± 19 vs. 592 ± 21 mg), soleus (111 ± 8 vs. 118 ± 4 mg), and gastrocnemius (1,506 ± 41 vs. 1,600 ± 79 mg) muscle weights did not differ between groups. Gastrocnemius dry/wet ratio, measured to exclude the possibility that tissue fluid accumulation may have masked uremic muscle wasting, did not differ between the two groups (0.23 ± 0.009 vs. 0.23 ± 0.004). Unilateral plantaris muscle WO for 7 days induced a similar and significant increase in muscle weight in CKD and SN rats compared with their respective nonloaded contralateral plantaris muscles.

BCAA and insulin levels. Plasma leucine levels, obtained 3 days before euthanization and measured chromatographically, did not differ significantly between the CKD and SN rats (91 ± 9 vs. 128 ± 19 nmol/ml). At euthanization, total BCAA levels measured by ELISA were similar in the two saline-treated groups and, 30 min following a leucine gavage, rose to comparable levels (Table 2). We have taken the increase in total BCAA levels to reflect an increase in leucine levels. Fasting plasma insulin levels were similar in the CKD-Sal and SN-Sal groups. Following a leucine gavage the insulin levels in the CKD-Leu and SN-Leu groups did not differ significantly from the levels in the corresponding saline-treated group (Table 2). This differs from the report by Anthony et al. (1), who described a threefold increase in insulin postleucine, and may reflect our use of ketamine-xylazine for anesthesia, which suppresses insulin levels (49).

Levels of signaling proteins in nonloaded plantaris muscle of SN and CKD rats. The levels of signaling proteins that participate in the mTOR signaling pathway, namely mTOR, 4E-BP1, S6K1, and rpS6, did not differ significantly between the two groups (Fig. 1), nor did the levels of STAT5 protein (100 ± 6 vs. 93 ± 2 arbitrary units).

Impact of leucine on signal transduction in nonloaded plantaris muscle of SN and CKD rats. Consistent with earlier reports (16, 32) and depicted in Figs. 2 and 3, administration of leucine to SN-Leu rats activated the mTOR signaling pathway involved in translation initiation of protein synthesis (16, 32). As shown in Fig. 2, phospho-mTOR levels in the saline-treated SN-Sal and CKD-Sal groups, taken to reflect basal levels, were similar. Following oral leucine, phospho-mTOR levels increased significantly and similarly, approximately threefold, in both groups. In contrast, basal phosphorylation of total 4E-BP1 in the CKD-Sal group was modestly lower than in the SN-Sal group (~68% of the SN value, P < 0.05). When leucine was given to CKD-Leu rats, phosphorylation of total 4E-BP1 increased significantly, but the levels reached were approximately one-third less than in the SN-Leu group (P < 0.05). Activation of mTOR results in phosphorylation of 4E-BP1 at multiple sites, including at Thr70, a site that appears to play the most important role in mediating the dissociation of 4E-BP1 from eIF4E (54). The phospho-4E-BP1 antibody used in this study detects only the 4E-BP1 forms phosphorylated at Thr70. When phosphorylated 4E-BP1 migrates in an electrophoretic gel, it separates into three to four bands according to the extent of phosphorylation at different sites (9). This was evident in the leucine-treated CKD and SN groups (Fig. 2A). Taking the above data together, it appears that 4E-BP1 phosphorylation is modestly attenuated in CKD, and since mTOR phosphorylation is unaffected, this suggests a downstream defect in 4E-BP1 phosphorylation and/or accelerated dephosphorylation or perhaps altered cross-talk from other signaling pathways.

The impact of CKD on the phosphorylation of S6K1 and its substrate rpS6 is shown in Fig. 3. S6K1 phosphorylation in
saline-treated CKD-Sal and SN-Sal rats was similar. Following leucine treatment, S6K1 phosphorylation increased significantly in both the CKD-Leu and the SN-Leu groups, with a greater increase in the CKD group (8-fold vs. 2-fold, \( P < 0.05 \)). Of note, basal phosphorylation of rpS6 was markedly suppressed in the CKD-Sal group, averaging only 10% of the SN-Sal value. However, leucine stimulated rpS6 phosphorylation in the CKD-Leu group to a level comparable with that observed in the SN-Leu group. This response in CKD despite the low basal phosphorylation may conceivably reflect the heightened phosphorylation of its upstream mediator S6K1. Taken together, it seems that leucine can effectively stimulate this arm of the mTOR signaling pathway despite attenuation of basal rpS6 phosphorylation. Thus abnormalities in the mTOR signaling pathway are evident in CKD even before the appearance of muscle atrophy. These include modestly suppressed basal and leucine-induced 4E-BP1 phosphorylation and severely suppressed basal rpS6 phosphorylation.

We also examined the effect of leucine on Akt phosphorylation, a kinase upstream of mTOR that is activated by ligands such as IGF-I and insulin and that in turn stimulates mTOR phosphorylation (35). Leucine failed to stimulate phosphorylation of Akt in the SN-Leu and CKD-Leu groups (Fig. 4), and taken together with our finding that insulin levels did not increase significantly after a leucine load, this is consistent with reports that leucine can stimulate mTOR signaling independent of insulin (1, 13, 25, 52).

Impact of WO on mTOR signaling. It is well established that an increase in mechanical load activates the mTOR signaling pathway in skeletal muscle (50), even in CKD (11, 56). In the present study, we observed that sustained WO of the plantaris muscle induced significant increases in the levels of phosphorylated mTOR, 4E-BP1, S6K1, and rpS6 in the SN rats (Figs. 5 and 6). In CKD, phosphorylation of the same four signaling proteins increased significantly after WO, and whereas the phospho-mTOR level reached was lower than in the SN group, phosphorylated 4E-BP1, S6K1, and rpS6 increased to values comparable with the SN group. Interestingly, WO stimulated a significant increase in the total protein levels of all four signaling proteins in SN and CKD rats (Figs. 5 and 6), although the 4E-BP1 and S6K1 levels reached were significantly lower in CKD.
Impact of leucine on signal transduction in work-overloaded hypertrophied plantaris muscle of SN and CKD rats. Since in normal humans oral leucine augments acute resistance exercise-induced mTOR signaling (16, 22), we tested whether leucine enhances mTOR signaling in chronically work-overloaded skeletal muscle and whether the response is affected in CKD. These results are shown in Figs. 7 and 8, which compare the relative phosphoprotein levels in the SN and CKD work-overloaded groups treated with either saline or leucine. For ease of comparison the results are expressed as a percent of the mean WO SN-Sal group assigned a value of 100. As depicted in Fig. 7, leucine did not alter mTOR phosphorylation in the SN group but did activate phosphorylation in the CKD group. Total 4E-BP1 phosphorylation increased similarly and significantly in both WO groups, and a clear shift in the phosphorylated moiety is evident in the Western immunoblot. As shown in Fig. 8, following leucine treatment S6K1 phosphorylation increased approximately fivefold in the WO SN-Leu group, but the response was attenuated significantly in the WO CKD-Leu group, with an approximately threefold increase. Of note, leucine failed to stimulate phosphorylation of the S6K1 substrate rpS6 in WO SN-Leu and CKD-Leu muscle, and rpS6 phosphorylation was significantly lower in the latter group (Fig. 8). Thus it appears that leucine enhanced signaling through the WO-activated mTOR signaling pathway in muscle of control animals by increasing 4E-BP1 and S6K1 phosphorylation. In CKD, leucine also induced an increase in phosphorylation of both of these proteins, although the S6K1 phosphorylation response was somewhat attenuated.

DISCUSSION
Skeletal muscle protein loss, a common complication of advanced CKD, may in part be accounted for by the impact of the uremic environment on the ability of muscle to respond to anabolic factors and to utilize nutrients (38). mTOR signaling plays a major role in integrating various nutrient, hormonal, energy, and mechanical stimuli that promote protein synthesis (35), and its activation by insulin/IGF-I is impaired in CKD (4). In this investigation, we set out first to determine whether activation of the mTOR-mediated anabolic signaling cascade by the BCAA leucine is intact in CKD and second whether leucine can enhance mechanically induced mTOR signaling in uremia. We studied 5/6-nephrectomized CKD and SN control rats with surgically induced unilateral plantaris muscle WO, a model of resistance exercise that also allows the examination of the contralateral nonloaded muscle (10, 55). CKD and SN rats were gavaged once with leucine or saline, the latter taken to reflect basal values, and then studied. Plasma creatinine levels were elevated threefold in the CKD group, and these animals gained less weight than the control SN rats consuming a comparable amount of food. Nevertheless, despite the attenuated moity is evident in the Western immunoblot. As shown in Fig. 8, following leucine treatment S6K1 phosphorylation increased approximately fivefold in the WO SN-Leu group, but the response was attenuated significantly in the WO CKD-Leu group, with an approximately threefold increase. Of note, leucine failed to stimulate phosphorylation of the S6K1 substrate rpS6 in WO SN-Leu and CKD-Leu muscle, and rpS6 phosphorylation was significantly lower in the latter group (Fig. 8). Thus it appears that leucine enhanced signaling through the WO-activated mTOR signaling pathway in muscle of control animals by increasing 4E-BP1 and S6K1 phosphorylation. In CKD, leucine also induced an increase in phosphorylation of both of these proteins, although the S6K1 phosphorylation response was somewhat attenuated.
uated weight gain, muscle mass did not differ between the two groups, and it is likely that the lower body weight reflects reduced fat mass, a component of the protein energy-wasting syndrome often complicating advanced CKD (34).

The first part of this study was directed at examining the impact of CKD on leucine-stimulated mTOR signaling in nonloaded skeletal muscle. Consistent with an insulin-independent action (22), we found that leucine did not increase Akt phosphorylation. However, leucine did stimulate downstream phosphorylation of mTOR, 4E-BP1, S6K1, and rpS6 in both groups of rats, although some significant abnormalities in the mTOR anabolic signaling pathway were evident in CKD, which we suggest likely predisposes and contributes to muscle wasting as renal failure advances. In CKD we found that basal mTOR phosphorylation was intact, but 4E-BP1 phosphorylation was depressed significantly by approximately one-third. Following an oral leucine load, mTOR phosphorylation increased threefold in both the SN and CKD rat muscle. 4E-BP1 phosphorylation also increased significantly, but the final level achieved was lower in CKD, presumably in part because of the lower basal level. In contrast, basal phosphorylation of another mTOR target, namely S6K1, was similar in the CKD and SN groups and, following the leucine load phosphorylation, increased significantly more in CKD, eight- vs. twofold. Of note, basal phosphorylation of rpS6, a substrate of S6K1, was reduced significantly in CKD, averaging only 10% of the SN value. This protein plays a critical role in translational control of protein synthesis and cell proliferation (39). Despite attenuated basal levels, leucine stimulated rpS6 phosphorylation to a level comparable with the response in the SN group. This CKD response may conceivably reflect the heightened leucine-induced phosphorylation of its upstream mediator S6K1. Thus, despite depressed basal rpS6 phosphorylation, leucine effectively stimulates this arm of the mTOR signaling pathway in

Fig. 6. Impact of WO on the level of phosphorylated and total S6K1 and rpS6 protein in plantaris muscle of SN and CKD rats. A: Western blot analysis of nonloaded control (non-WO) and contralateral WO muscle from SN-Sal and CKD-Sal rats. Muscle was collected after 7 days of unilateral WO. Pooled samples (6/group) were run in duplicate. B: phosphorylated protein levels corrected for loading are expressed as a percent of mean non-WO SN-Sal group level assigned a value of 100. Results are means ± SE of 6 individual samples/group. *P < 0.05, paired t-test, non-WO vs. contralateral WO muscle. #P < 0.05, unpaired t-test SN-WO vs. CKD-WO muscle.

Fig. 7. Impact of leucine on mTOR and 4E-BP1 phosphorylation in work-overloaded plantaris muscle of SN and CKD rats. A: Western immunoblots of WO muscle from CKD and SN rats gavaged with Sal or Leu. Pooled samples (6/group) were assayed in duplicate. B: phosphoprotein levels corrected for specific protein levels are expressed as a percent of mean work-overloaded SN-Sal group value assigned a value of 100. Bars are means ± SE of 6/group. Different letters above bars indicate a difference between groups of P < 0.05.
CKD. Taken together with the partial stimulation of 4E-BP1 phosphorylation, this suggests that leucine-rich supplements may be useful in managing uremic muscle wasting, especially since serum leucine levels are commonly reduced in advanced CKD (8, 23) and protein synthesis is modulated by extracellular AA availability (6). In addition, leucine has known antiproteolytic properties (40, 58). Indeed, in a small double-blinded study, the nutritional status of malnourished hemodialysis patients improved when given oral BCAA supplements (26).

In the second part of this study directed at testing whether in CKD leucine can enhance the anabolic signaling seen in response to an increase in skeletal muscle mass, novel findings were also observed. First, expanding on earlier studies (11, 56), we noted that sustained WO for 7 days resulted in an increase in the levels of mTOR, 4E-BP1, S6K1, and rpS6 protein and phosphoprotein levels in the hypertrophied plantaris muscle of control SN and CKD rats. However, compared with the control animals, WO-induced mTOR phosphorylation was moderately attenuated in CKD. But this was not severe enough to impair the downstream phosphorylation of 4E-BP1, S6K1, and rpS6 or the levels of these phosphorylated proteins increased to that observed in the WO control animal muscle. Total protein available for phosphorylation also increased in response to WO in both groups of animals but modestly less so in CKD.

When leucine was given to the control SN rats, signal transduction through the WO-activated mTOR pathway was augmented even further. Phosphorylation of 4E-BP1 and S6K1 increased significantly, although unlike the positive response in the nonloaded muscle, rpS6 phosphorylation did not change significantly. This lack of change may conceivably indicate that phosphorylation had already reached a maximum. When given to the CKD rats, leucine caused a further increase in mTOR and 4E-BP1 phosphorylation in the WO muscle that reached levels seen in the WO SN-leucine treated group. In contrast, although leucine increased S6K1 phosphorylation significantly, by threefold, the response was attenuated in CKD, and again, unlike the response in nonloaded muscle, leucine did not alter rpS6 phosphorylation, and the phospho-rpS6 levels were significantly lower than in the WO SN-leucine treated group. Taken together, although less effective than in normals, the largely positive response to leucine in rats with moderate CKD provides the rationale for studying whether in severe experimental CKD BCAA supplementation together with exercise is effective in maintaining or increasing muscle mass.

The pathomechanisms accounting for the perturbations in leucine-stimulated, mTOR-related signal transduction in CKD were not formally examined in this study. However, the signaling abnormalities cannot be attributed to the prevailing plasma insulin or leucine levels. Basal insulin levels were similar in the control and CKD rats and did not change significantly after a leucine load, nor did Akt phosphorylation increase. Similarly, basal plasma leucine levels did not differ between the two groups. After an oral leucine load, the plasma levels increased to comparable levels in both groups. This indicates that intestinal leucine absorption was intact in the CKD group and that over the period of study the levels were unaffected by any changes in leucine clearance/metabolism that may occur in kidney failure (7, 20). Delivery and transport of leucine into the muscle cells, regarded as a potential mechanism of uremic insulin resistance (17), did not appear to play a role, since mTOR phosphorylation in nonloaded muscle was unimpaired. Thus other mechanisms must be operative to account for the perturbations in events downstream of mTOR and may reflect impaired phosphorylation and/or perhaps more rapid dephosphorylation of signaling proteins (12) or perhaps dysregulated input from other signaling pathways.

Typically, uremia-related resistance to anabolic stimuli such as insulin/IGF-I and growth hormone arise because of factors such as acidosis, sepsis, inflammation, poor dietary intake, and unidentified circulating inhibitors (38). Among these factors, acidosis as a cause of the leucine resistance appears to be excluded since the serum bicarbonate levels in the CKD rats were within the normal range. Inflammation common in CKD (38) is an important cause of impaired signaling through the mTOR pathway (33), but the pattern of abnormalities in inflammation differs in part from that observed in this study. One major difference is that inflammation attenuates mTOR phosphorylation, which is intact in CKD. On the other hand, phosphorylation of 4E-BP1 and rpS6 is impaired in both inflammation and CKD. Overproduction of proinflammatory...
cytokines and glucocorticoids seems to play an important role when inflammation is present (35).

A limitation of this study was the absence of muscle wasting in the CKD rats. However, we believe that this model has uncovered novel information regarding the early appearance of signaling abnormalities in CKD and the response to leucine. Although alone the abnormalities appear to be insufficient to cause muscle wasting, they may nevertheless contribute to the development of muscle wasting if these abnormalities worsen as renal failure progresses and other abnormalities such as acidosis also come in to play. In support of this notion is the early appearance of insulin resistance in nondiabetic patients with mild renal dysfunction (28), for in advanced CKD insulin resistance plays an important role in the development of muscle wasting (38). Nevertheless, caution must be taken when extrapolating these findings obtained in a rodent model of CKD to the clinical situation. In this regard, it should also be kept in mind that the work-induced stimulus in the compensatory WO model of resistance exercise used in this study is continuous and may not in all respects reproduce the intermittent nature of the resistance exercise training performed by humans.

In conclusion, the findings presented above have established that chronic uremia impairs basal signal transduction through the mTOR pathway in non-work-loaded skeletal muscle at the level of 4E-BP1 and rpS6 phosphorylation and partially attenuates leucine-induced activation of this pathway. Since the mTOR signaling pathway plays a key anabolic role, we suggest that these abnormalities, detected in the absence of muscle wasting, may contribute to the eventual development of muscle wasting. In skeletal muscle undergoing compensatory hypertrophy, leucine did augment the work-induced activation of the mTOR pathway, but again the response was partially attenuated in CKD. Taking all this into account, the fact that leucine does enhance signal transduction through the mTOR signaling pathway in CKD, albeit not as effectively as in normal controls, leads us to suggest that there may be a role for leucine-rich supplements in the management of uremic muscle wasting. This approach would be analogous to the recommendation that leucine-rich supplements be provided for age-related wasting, another condition with partial resistance to leucine (31, 41). However, further studies in rats with more severe kidney failure and muscle wasting are first needed to provide proof of concept.

GRANTS
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DISCLOSURES
The authors have nothing to disclose.

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