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Acute uremia suppresses leucine-induced signal transduction in skeletal muscle

Kevin L. McIntire[1,2], Yu Chen[1,2], Sumita Sood[1,2] and Ralph Rabkin[1,2]

[1]Research Service, Veterans Affairs Palo Alto Health Care System (VAPAHCS), Palo Alto, California, USA and [2]Renal Division, Department of Medicine, Stanford University, Stanford, California, USA

Adequate nutrient intake in acute uremia is a key part of patient management especially as food utilization is usually impaired. Leucine is important as it comprises about one-fifth of essential amino acid needs and, apart from serving as a substrate, it directly activates the mTOR signaling pathway stimulating protein synthesis and inhibiting autophagy. Here we tested whether leucine activation of the mTOR signaling pathway in muscle is severely disrupted in acute uremia. Several abnormalities were identified in bilateral ureteral ligated (model of acute uremia) compared to sham-operated pair-fed control rats. Levels of several signaling proteins increased significantly while leucine-induced phosphorylation of mTOR and downstream proteins, 4e-BP1 and S6K1, was completely suppressed. Levels of LC3B-II, a specific autophagosomal membrane–associated protein used as a marker of autophagy, increased threefold in uremia. Furthermore, while leucine suppressed LC3B-II levels in controls, it failed to do so in uremic rats. Muscle IL-6 mRNA levels increased, while IGF-1 mRNA levels decreased in uremia. These findings establish that, in acute uremia, severe resistance to leucine-induced activation of the mTOR anabolic signaling pathway develops. Thus, leucine resistance, together with the reduction in IGF-1 and increase in IL-6 expression, may explain why the anabolic effect of nutritional therapy is diminished in acute uremic patients.

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Correspondence: *Ralph Rabkin, Research Service, Veterans Affairs Palo Alto Health Care System (VAPAHCS) (111R), 3801 Miranda Avenue, Palo Alto, California 94304, USA. E-mail: rabkin@stanford.edu*

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Severe acute kidney injury (AKI) is associated with a 45–56% intensive care unit mortality rate[1] and has negative effects on multiple tissues[2,3] including skeletal muscle, eventually leading to a reduction in muscle mass.[4] This wasting negatively affects skeletal and respiratory muscle strength and function, delaying recovery.[5] As in other wasting conditions, muscle atrophy in AKI arises because of decreased protein synthesis (PS)[6] and increased proteolysis,[7] especially through the ubiquitin–proteasome system (UPS) that degrades myofibrilar and short-lived proteins. Other proteolytic systems including autophagy, responsible for degradation of long-lived proteins and damaged organelles, as well as the calpain and caspase systems, may also be involved, but have been poorly studied in uremia.[7] Many factors contribute to wasting in AKI,[8–10] including anorexia, inflammation, and acidosis, the latter being an activator of the UPS. Also important are resistance to and/or deficiencies of anabolic hormones and nutrients, especially amino acids (AAs).[11–13] In this study, we focus on elucidating the mechanisms accounting for the insensitivity to the anabolic promoting actions of leucine, an abundant and important branched-chain AA (BCAA).

Leucine comprises ~20% of essential AA requirements,[14] and, in addition to serving as a substrate, it directly activates the multiprotein mammalian target of rapamycin complex 1 (mTOR) signaling pathway[15] that increases translation initiation and PS,[16] and suppresses autophagy.[17] After leucine ingestion, phosphorylated mTOR (p-mTOR) increases rapidly and in turn phosphorylates the eukaryotic translation initiation factor 4e–binding protein 1 (4e-BP1) and the serine/threonine protein kinase p70S6 kinase 1 (S6K1).[18] Phosphorylation of 4e-BP1, a translational repressor, leads to its release from eukaryotic translation initiation factor 4e, which is necessary for mRNA translation activation.[18,19] Phosphorylated S6K1 phosphorylates many proteins including 40S ribosomal protein S6 (rpS6), and PS increases.[19] Apart from stimulating anabolism, leucine inhibits proteolysis partly by suppressing autophagy[20] and possibly by suppressing the UPS.[21,22] Leucine also stimulates insulin secretion, which activates mTOR signaling via the phosphatidylinositol 3 kinase/Akt pathway, contributing to its anabolic properties.[23]

As in other catabolic states, uremic muscle wasting has largely been attributed to increased UPS activity.[24] However, autophagy, important for maintaining normal muscle mass,

can also account for a significant, although lesser, amount of muscle breakdown.[25,26] Increased autophagy has not been described in uremia. Autophagy is a complex process involving the formation of a vesicle, the autophagosome, that engulfs damaged cytosolic proteins, glycogen, and malfunctioning organelles. Thereafter, autophagosomes fuse with lysosomes and degradation proceeds. Autophagosome formation involves multiple proteins including ULK1, involved in the initiation of autophagosome formation, and microtubule-associated protein light chain 3 (LC3), involved in late-stage autophagosome formation. Ulk1 is directly regulated by mTOR (inhibitory) and 5′ adenosine monophosphate activated protein kinase (AMPK) (stimulatory), and it appears that leucine inhibits autophagy through p-mTOR-mediated disruption of p-AMPK activation of Ulk1.[20] LC3 undergoes processing to form LC3-I and then LC3-II, a specific autophagosomal membrane–associated protein. The LC3B-II isoform correlates with autophagic vesicle number and is often used as an indirect measure of autophagy, but with some caveats.[27,28]

Resistance to leucine-induced signaling develops in catabolic states, for example, sepsis and cancer, manifested by reduced leucine-stimulated phosphorylation of mTOR and its downstream targets and consequently impaired PS.[29] Furthermore, we recently uncovered a state of partial leucine resistance in rats with moderate nonacidotic chronic kidney disease (CKD).[12] In this study, we have tested the hypothesis that leucine activation of the mTOR pathway, which regulates PS and degradation in skeletal muscle, is markedly disrupted in severe acute acidotic uremia. If true, this would represent a potential cause of the reduced effectiveness of AA supplementation in AKI.[30]

RESULTS

Anthropometrics and plasma biochemistry

Serum creatinine and urea nitrogen increased markedly after ureteral ligation, whereas bicarbonate levels decreased (Table 1). Both AKI and control (CON) groups lost body weight with a smaller loss in AKI, which is likely attributable to fluid retention (Table 1). Both plantaris muscles were weighed together, and there were no differences between groups. BCAA levels in saline (Sal)-treated AKI and CON groups taken to reflect basal values did not differ statistically (Table 2). After the leucine (Leu) gavage, BCAA levels in CON-Leu and AKI-Leu groups increased to similar levels, and the increase above the corresponding saline-treated group was taken to reflect an increase in plasma leucine level. Fasting insulin levels were ninefold higher in the AKI-Sal group versus the CON-Sal group (Table 2), consistent with loss of renal function.[31,32] After the leucine gavage, insulin levels increased twofold in the CON-Leu group, but did not reach statistical significance. In the AKI-Leu group, insulin levels decreased by half, to a value not significantly different from the AKI-Sal value, a response consistent with an *in vitro* study.[33]

Table 1 | Serum biochemistry and anthropometrics

	Control	AKI
Number of rats	12	12
Creatinine (mg/dl)	0.3 ± 0.02	5.33 ± 0.09**
Urea nitrogen (mg/dl)	14 ± 0.7	206 ± 4.8**
Bicarbonate (mmol/l)	23 ± 0.5	17 ± 0.4**
Initial body weight (g)	240 ± 4	241 ± 4
Ending body weight (g)	215 ± 5#,†	231 ± 4#,†
Left and right plantaris muscles combined (mg)	440 ± 12	440 ± 8

Abbreviation: AKI, acute kidney injury.
#P < 0.05 versus control; **P < 0.001 versus control; †P < 0.001 versus initial body weight.
Values are mean ± s.e.

Basal levels of signaling proteins and phosphorylated proteins in plantaris muscle

In AKI-Sal rats, basal levels of several mTOR pathway proteins were significantly increased compared with CON-Sal values (Figure 1). Specifically, mTOR protein increased by 60%, 4e-BP1 by 100%, and S6K1 by 50%. As detailed later, Akt levels also increased significantly, whereas other proteins, namely rpS6, AMPK, and STAT5, were unchanged. The latter was used as a loading control. In keeping with the increase in respective signaling protein levels, average basal phosphorylated mTOR, 4e-BP1, and S6K1 levels increased in the AKI-Sal groups (Figure 1c), although only the p-mTOR and p-4e-BP1 levels reached statistical significance. Relative phosphorylation (phospho protein/specific protein) of these same proteins did not differ between groups, indicating that basal efficiency of protein phosphorylation was unaltered in AKI (Figure 1d).

Leucine-stimulated anabolic signal transduction in plantaris muscle

Leucine administration activated mTOR signaling in the CON-Leu rats. Phosphorylated mTOR, 4e-BP1, and S6K1 levels increased significantly above CON-Sal group values (Figure 2): average increase of 55, 182, and 179%, respectively. In contrast, the leucine effect was severely compromised in AKI, and leucine failed to increase p-mTOR, p-4e-BP1, or p-S6K1 levels. Note that 4eBP1 is phosphorylated at multiple sites and separates into three bands (α, β, and γ) in a one-dimensional electrophoretic gel according to the extent of phosphorylation.[34] The hyper-phosphorylated γ band was quantified, which is considered the fully active isoform. Also note that there was no change in the loading control protein STAT5 (signal transducer and activator of transcription 5). Unexpectedly, leucine increased the p-rpS6 levels in the AKI-Leu group, although on average less than in the CON-Leu group (Figure 3). As rpS6 is phosphorylated by p-S6K1, unresponsive to leucine in AKI (Figure 2), this suggests that leucine may be activating an alternative pathway to rpS6 in AKI.

With respect to Akt, similar to mTOR, 4e-BP1, and S6K1, basal protein levels increased significantly by 40% (Figure 4). An interesting phosphorylation response to leucine and AKI was evident (Figure 4). But keeping in mind that Akt is activated by phosphorylation on two critical sites, threonine