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ORIGINAL ARTICLE

Sepsis-induced changes in amino acid transporters and leucine signaling via mTOR in skeletal muscle

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Abstract The present study tested the hypothesis that sepsis-induced leucine (Leu) resistance in skeletal muscle is associated with a down-regulation of amino acid transporters important in regulating Leu flux or an impairment in the formation of the Leu-sensitive mTOR–Ragulator complex. Sepsis in adult male rats decreased basal protein synthesis in gastrocnemius, associated with a reduction in mTOR activation as indicated by decreased 4E-BP1 and S6K1 phosphorylation. The ability of oral Leu to increase protein synthesis and mTOR kinase after 1 h was largely prevented in sepsis. Sepsis increased CAT1, LAT2 and SNAT2 mRNA content two- to fourfold, but only the protein content for CAT1 (20 % decrease) differed significantly. Conversely, sepsis decreased the proton-assisted amino acid transporter (PAT)-2 mRNA by 60 %, but without a coordinate change in PAT2 protein. There was no sepsis or Leu effect on the protein content for RagA-D, LAMTOR-1 and -2, raptor, Rheb or mTOR in muscle. The binding of mTOR, PRAS40 and RagC to raptor did not differ for control and septic muscle in the basal condition; however, the Leu-induced decrease in PRAS40·raptor and increase in RagC·raptor seen in control muscle was absent in sepsis. The intracellular Leu concentration was increased in septic muscle, compared to basal control conditions, and oral Leu further increased the intracellular Leu concentration similarly in both control and septic rats. Hence, while alterations in select amino acid transporters are not associated with development of sepsis-induced Leu resistance, the Leu-stimulated binding of raptor with RagC and the recruitment of mTOR/raptor to the endosome-lysosomal compartment may partially explain the inability of Leu to fully activate mTOR and muscle protein synthesis.

Keywords Sepsis · Protein synthesis · mTOR · Amino acid transporters · Rag GTPases · PRAS40 · Ragulator

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Introduction

The erosion of lean body mass (LBM) resulting from prolonged sepsis and inflammation is mediated not only by a reduction in the basal rate of muscle protein synthesis but also an impaired response to many anabolic stimuli, including growth factors (insulin and insulin-like growth factor-I) and nutrients (amino acids, leucine) (Lang and Frost 2004, 2006; Lang et al. 2010). Both these sepsis-induced defects are in part mediated by an attenuation of the regulatory kinase mTOR (mammalian target of rapamycin) which impairs cap-dependent mRNA translation (Frost and Lang 2011), although the specific mechanism for this impairment remains to be determined. Regardless of its etiology, the loss of LBM in sepsis remains a clinical concern as it compromises recovery and increases morbidity in the chronically ill patient population (Callahan and Supinski 2009; Blackburn and Bistrian 1976).

The ability of the essential amino acid leucine to increase mTOR activity is central to regulating global protein synthesis and maintaining cellular protein homeostasis (Dodd and Tee 2012). Several mechanisms have been proposed for leucine activation of mTOR (Avruch et al. 2009; Jewell et al. 2013), which could conceivably function independently or in combination to promote anabolic signal transduction. Amino acid transporters have been

implicated in the regulation of mTOR by at least two mechanisms. First, the classical transport function, which individually or coupled with other transporters provides a conduit for extracellular leucine to enter the cell or for the redistribution of leucine within the cell (Verrey 2003; Hatzoglou et al. 2004). Alternatively or additionally, some transporters [sodium-coupled neutral amino acid transporters (SNAT2/slc38A2) and proton-assisted amino acid transporters (PAT1-4; slc36A)] have been suggested to have a nutrient signaling component thereby functioning as a "transceptor" (Hundal and Taylor 2009; Ogmundsdottir et al. 2012). However, characterization of sepsis-induced changes in the muscle expression of various amino acid transporters is lacking. Once inside the cell, several models have been proposed whereby leucine stimulates the translocation and activation of mTOR to the late endosome–lysosome where it interacts with a number of proteins, including the Ragulator, vacular (v)-ATPase, and the Rag-GTPases (Sancak et al. 2010; Zoncu et al. 2011). However, how sepsis impacts these various components of leucine signal transduction to mTOR has not been fully assessed. Hence, the present study tested the hypothesis that sepsis-induced leucine resistance in skeletal muscle is associated with a down-regulation of amino acid transporters important in regulating leucine flux or impairment in the formation of the leucine-sensitive mTOR–Ragulator complex.

Materials and methods

Animal protocols

Male-specific pathogen-free Sprague–Dawley rats (Charles Rivers Breeding Lab, Cambridge, MA) were quarantined for 1 week and at the time of surgery weighed 300–325 g (11–12 weeks of age). Rats were housed under controlled environmental conditions, 12 h light/12 h dark, 21–22 °C, and 30–70 % humidity; no environment enrichment was provided. Rats were housed 3 per cage (wire-bottom) and provided Teklad Global 2019 [calories from protein (23 %), fat (22 %) and carbohydrate (55 %); Harlan Teklad, Boston, MA] and tap water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine (protocol #43186) and adhered to the National Institutes of Health (NIH) guidelines for the use of experimental animals.

Induction of sepsis

Polymicrobial peritonitis was produced by cecal ligation and puncture (CLP), as previously described (Lang and Frost 2004, 2006) in fed animals. This model was selected because it produces a septic state mimicking that seen in humans, and is recognized as an appropriate small animal model of peritonitis. Rats were anesthetized with isoflurane (3 % induction +2–3 % maintenance; Abbott Laboratories, North Chicago, IL). The abdominal hair was clipped and the skin cleaned with povidone–iodine prior to performing a midline laparotomy. The cecum was isolated, ligated, punctured twice with an 18-gauge needle, and a small amount of cecal material extruded to ensure patency. The cecum was returned to the abdomen, the muscle incision closed with 4–0 surgical suture (Ethicon, Inc., Somerville, NJ), and the skin incision closed with metal wound clips. Lidocaine (Abbott Laboratories), 2–3 drops, was applied to the wound for analgesia. After surgery, rats were housed individually in shoe-box cages with corn-cobb bedding. Rats received 10 mL of 0.9 % sterile saline (37 °C) containing buprenorphine (0.05 mg/kg; Reckitt Benckiser Pharmaceuticals, Richmond, VA) administered subcutaneously every 12 h. Sham control animals experienced the same surgical procedures, but the cecum was not ligated or punctured. Before surgery, animals had unrestricted access to food and water. However, as food consumption in septic rats is nominal during the first 24 h post-CLP, food was withheld from all animals so metabolic differences between groups would be independent of differences in caloric intake.

A separate group of control and septic rats were administered an oral gavage of either leucine (1.35 g/kg BW) or saline, and skeletal muscle excised 60 min thereafter. The dose of leucine was selected based on prior studies demonstrating maximal stimulation of muscle protein synthesis and phosphorylation of 4E-BP1 and S6K1 (Crozier et al. 2005). Timing of the muscle sample after leucine administration was selected based on previous studies indicating that leucine increases the mRNA expression of several amino acid transporters (Drummond et al. 2010, 2012). This model of sepsis has been previously demonstrated to decrease basal and leucine-stimulated muscle protein synthesis (Kazi et al. 2011; Lang and Frost 2004, 2006; Lang et al. 2005). In vivo studies were repeated on three separate occasions with each study including all four experimental groups.

In vivo protein synthesis

The in vivo rate of protein synthesis in the gastrocnemius and plantaris complex (hereafter referred to as muscle) was determined ~24 h after induction of sepsis using the flooding-dose technique, as detailed previously (Vary and Lang 2008). Rats were anesthetized with isoflurane as described above and a catheter inserted in the carotid artery. Subsequently, L-[2,3,4,5,6-3H]phenylalanine (Phe; 150 mM, 30 μCi/ml; 1 ml/100 g body weight [BW]) was

administered as a bolus injection into the jugular vein. Serial arterial blood samples were drawn at 2, 6 and 10 min after Phe injection for measurement of Phe concentration and radioactivity. Immediately after the final blood sample, skeletal muscle was excised and a portion freeze-clamped; remaining fresh muscle was directly homogenized. Blood was centrifuged and plasma was collected. All tissue and plasma samples were stored at −80° C. A portion of the frozen muscle was used to estimate the global rate of incorporation of [^{3}H]Phe into protein, exactly as previously described (Vary and Lang 2008).

Western blot analysis and immunoprecipitation

Fresh tissue was homogenized (Kinematic Polytron; Brinkmann, Westbury, NY) in ice-cold homogenization buffer consisting of (in mmol/L): 20 HEPES (pH 7.4), 2 EGTA, 50 sodium fluoride, 100 potassium chloride, 0.2 EDTA, 50 β-glycerophosphate, 1 DTT, 0.1 phenyl-methane-sulphonylfluoride, 1 benzamidine, and 0.5 sodium vanadate. The protein concentration was quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and equal amounts of total protein per sample were subjected to standard SDS-PAGE (Kazi et al. 2011; Lang and Frost 2004, 2006, Lang et al. 2005, 2010). All antibodies were purchased from Cell Signaling Technology (Beverly, MA) unless otherwise noted. As an index of mTOR kinase activity, Western blot analysis was performed for total and phosphorylated (T37/46) 4E-BP1 (Bethyl Laboratories, Montgomery, TX) as well as total and phosphorylated (T389) S6K1 which are downstream mTOR substrates. To assess activation of 4E-BP1, the amount of eIF4G bound to immunoprecipitated eIF4E was quantitated; activation of S6K1 was determined by immunoblotting for total and phosphorylated ribosomal protein S6 (S240/244). Total and Ser473 phosphorylated AKT as well as total and Thr246 phosphorylated PRAS40 (proline-rich AKT substrate 40) were assessed as upstream regulators of PRAS40 and its binding to raptor. The relative expression of other proteins was determined by antibodies for CAT1 (cationic amino acid transporter 1; SLC7A1), LAT2 (L-type amino acid transporter 2; SLC7A5) and SNAT2 (sodium-coupled neutral amino acid transporter 2; SLC38A2) (Santa Cruz Biotechnology, Dallas, TX). Also, PAT-1 and PAT-4 (Santa Cruz Biotechnology) and PAT2 (Millipore, Billerica, MA) as well as LAMTOR (late endosomal/lysosomal adaptor, MAPK and mTOR activator) 1 and 2, mTOR, raptor and Rheb were determined by Western blotting. All lanes were normalized for protein loading and blotted for tubulin or other appropriate loading control. Blots were developed with enhanced chemiluminescence (ECL) Western blotting reagents (Supersignal Pico, Pierce Chemical, Rockford, IL). Dried blots were exposed to X-ray film to achieve a signal within the linear range and film was then scanned (Microtek ScanMaker IV; Cerritos,CA) and quantified using Scion Image 3b software (Scion Corporation, Frederick, MD).

The eIF4E·eIF4G complexes were quantified by immunoprecipitation (Kazi et al. 2011). Briefly, eIF4E was immunoprecipitated from aliquots of supernatants using an anti-eIF4E monoclonal antibody. Antibody–antigen complexes were collected using magnetic beads, subjected to SDS-PAGE, and finally transferred to a PVDF membrane. Blots were incubated with a mouse anti-human eIF4E antibody, rabbit anti-rat 4E-BP1 antibody, or rabbit anti-eIF4G antibody. Also, to investigate protein–protein interactions within mTOR complex 1 (mTORC1), fresh muscle was also homogenized in CHAPS buffer (pH 7.5) composed of (in mM): 40 HEPES, 120 NaCl, 1 EDTA, 10 pyrophosphate, 10 β-glycerophosphate, 50 NaF, 1.5 sodium vanadate, 0.3 % CHAPS and 1 protease inhibitor cocktail tablet. The homogenate was clarified by centrifugation and an aliquot of the supernatant was combined with anti-Raptor antibody and immune complexes were isolated with goat anti-rabbit BioMag IgG (PerSeptive Diagnostics, Boston, MA) beads. The beads were collected, washed with CHAPS buffer, precipitated by centrifugation and subjected to SDS-PAGE as described above. All blots were then developed with ECL and the autoradiographs were quantified as above and previously described (Kazi et al. 2011; Lang and Frost 2004, 2006).

RNA extraction and real-time quantitative PCR

Total RNA was extracted using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) and RNeasy mini kit (Qiagen, Valencia, CA) following manufacturers' protocols. Tissue was homogenized in Tri-reagent followed by phenol/chloroform extraction according to the manufacturer's instruction. An equal volume of 70 % ethanol was added to the aqueous phase and the mixture was loaded on a Qiagen mini-spin column. The Qiagen mini-kit protocol was followed from this step onwards including the on-column DNase I treatment to remove residual DNA contamination. RNA was eluted from the column with RNase-free water and an aliquot was used for quantitation (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA). Quality of the RNA was analyzed on a 1 % agarose gel. Total RNA (1 μg) was reversed transcribed to cDNA using superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) in a total reaction volume of 20 μl following instructions from the manufacturer. Real-time quantitative PCR was performed on 1–2 μl of the reversed transcribed reaction mix in a StepOnePlus system using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for the following: CAT-1 (slc7a1, NM_013111.2); SNAT-2 (slc38a2,

NM_181090.2); LAT-2 (slc3a2, NM_019283.1); PAT-1 (slc36a1, NM_130415.1); PAT-2 (slc36a2, NM_139339.1); PAT4 (slc36a4, NM_001108127.1); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_017008.3). The comparative quantitation method 2-ΔΔCt was used in presenting gene expression of target genes in reference to the endogenous control.

Plasma concentrations

The plasma insulin concentration was determined by ELISA (Alpco; Salem, NH) and the leucine and glutamine concentrations in plasma and muscle were determined using reverse-phase high-pressure liquid chromatography after precolumn derivatization of amino acids with phenylisothiocyanate (Vary and Lang 2008).

Statistics

Data for each condition are summarized as mean ± standard error of the mean (SEM) where the number of rats per treatment is indicated in the legend to the figure or table. Statistical evaluation of the data was performed using 1-way ANOVA with post hoc Student–Neuman–Keuls test when the interaction was significant. Differences between groups were considered significant at $P < 0.05$. GraphPad Prism version 5.0 (GraphPad software, La Jolla, CA) was used for statistical analysis.

Results

Figure 1 provides data related to sepsis-induced changes in protein synthesis under the basal condition and following leucine stimulation. Sepsis decreased global protein synthesis 35 % in gastrocnemius (predominantly fast-twitch), but not soleus (predominantly slow-twitch) (Fig. 1a, b, respectively). This sepsis-induced decrease in gastrocnemius was accompanied by an impaired mTOR activity as indicated by the 45–55 % reduction in phosphorylation of 4E-BP1 and S6K1, both downstream substrates for mTOR (Fig. 1c, d, respectively). As the formation of the active eIF4E·eIF4G complex is proportional to the extent of 4E-BP1 phosphorylation, the sepsis-induced reduction in eIF4E·eIF4G binding (65 %; Fig. 1e) is consistent with the observed reduction in phosphorylated 4E-BP1. Likewise, the sepsis-induced reduction in the phosphorylation of S6 (35 %; Fig. 1f) is consistent with the above-mentioned decreased S6K1 activity. In nonseptic control rats, leucine increased protein synthesis in gastrocnemius (but not soleus) and this was associated with increased phosphorylation of 4E-BP1, S6K1 and S6 as well as an increased formation of the eIF4E·eIF4G complex (Fig. 1). This typical anabolic response to leucine was essentially absent in gastrocnemius from septic rats.

Amino acid transporters are important for regulating the intracellular concentration of leucine and other amino acids; hence, we assessed the mRNA and protein content for various transporters in gastrocnemius. In general, there was considerable discordance between mRNA and protein content determined for these transport proteins. For example, in the basal condition, sepsis increased CAT1 (2.7-fold), LAT2 (5.3-fold) and SNAT2 (2.4-fold) mRNA content (Fig. 2a, c, e). However, no coordinate up-regulation of these transporters at the protein level was detected (Fig. 2b, d, f). In contradistinction to the mRNA data, Western analysis indicated sepsis decreased muscle CAT1 and LAT2 protein content under basal conditions (25 and 32 %, respectively), but did not alter SNAT2 protein content. The mRNA expression for CAT1, LAT2 and SNAT2 did not differ between control and septic rats 1 h after oral leucine administration. However, leucine decreased the protein content 25–30 % for CAT1 and LAT2 in muscle from controls but not septic rats.

There was no sepsis effect on PAT1 or PAT4 mRNA content, but PAT2 mRNA was decreased by 60 % in muscle from septic rats (Fig. 3a–c). In contrast, the PAT2 *protein* content did not differ between control and septic muscle (Fig. 3d). There was also no sepsis-induced change in the muscle protein content for PAT1 and PAT4. Finally, leucine did not alter either mRNA or protein content for any of the PATs in either control or septic muscle.

There was no sepsis and/or leucine effect for total mTOR, raptor, Rheb, RagA, RagB, RagC, RagD, LAMTOR1 or LAMTOR2 in the whole muscle homogenate (data not shown). Next, raptor was immunoprecipitated and the binding of several interacting proteins assessed (Fig. 4). There was no sepsis- or leucine-induced change in the binding of mTOR to raptor (Fig. 4a). Likewise, in the basal condition the binding of PRAS40 and RagC to raptor did not differ in muscle from control and septic rats. However, the binding of PRAS40 to raptor was decreased ~20 % (Fig. 4b) and the binding of RagC to raptor increased ~50 % (Fig. 4c) in control muscle. These reciprocal responses to leucine were not detected in septic rats.

As PRAS40 is a downstream substrate of AKT and phosphorylation of PRAS40 releases its binding and inhibitory effect on raptor, we next examined total and phosphorylated AKT and PRAS40. Sepsis decreased Ser473 phosphorylated AKT by 45 % and this change was independent of a change in total AKT (Fig. 5a, b). Leucine gavage did not alter AKT phosphorylation in muscle of either control or septic rats. Sepsis also decreased Thr246 phosphorylated PRAS40 by 35–40 %, compared to control values, in the basal condition (Fig. 5a, c). In contrast,

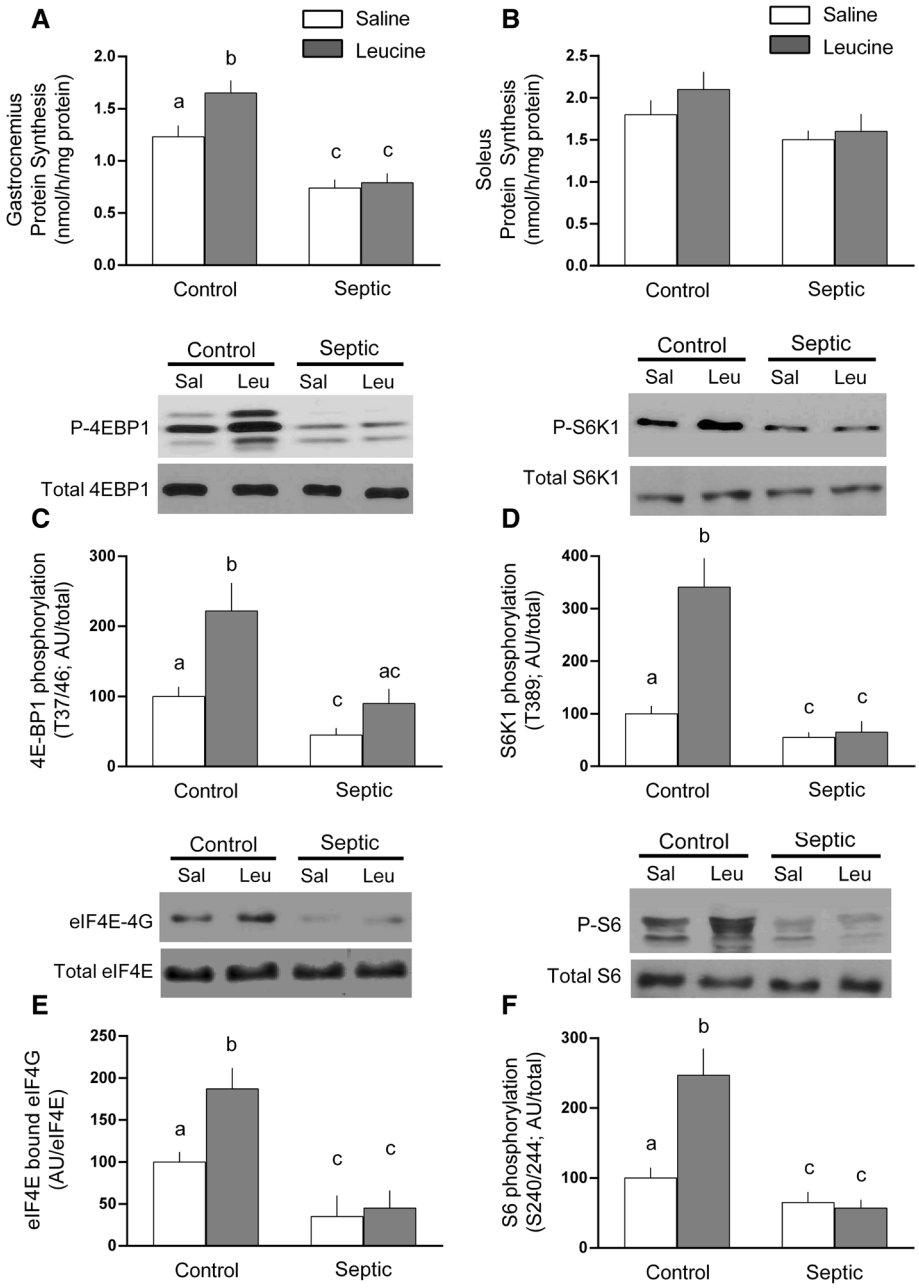

Fig. 1 Protein synthesis and mTOR signaling in gastrocnemius (**a**) and soleus (**b**) from control and septic rats under basal conditions or 1 h after oral administration of leucine (Leu) or saline (Sal). **c** and **d**, representative Western blots for phosphorylated and total 4E-BP1 and S6K1, endpoints of mTOR signaling in gastrocnemius. **e**, representative blot for binding of eIF4E with eIF4G, which is an indicator 4E-BP1 phosphorylation, determined on immunoprecipitated eIF4E. **f**, representative immunoblot for ribosomal protein S6, a downstream target of S6K1. For all immunoblots, data were quantitated for all rats and presented in *bar graphs* as mean ± SEM; $n = 8$, 9, 9 and 9, respectively, for the four experimental groups. Values having a different *superscript letter* (*a* vs. *b* vs. *c*) are statistically different ($P < 0.05$); values with the *same letter* are not significantly different

oral leucine increased PRAS40 phosphorylation in muscle from control rats by ~30 %, but no increase was detected in septic rats.

The plasma leucine concentration did not differ between control and septic rats in the basal condition or 1 h after oral administration of leucine; however, septic and control animals both exhibited ~8-fold increase in plasma leucine in response to leucine gavage (Fig. 6a). The intracellular leucine concentration was elevated 75 % in muscle from septic rats in the basal condition, compared to control values (Fig. 6b). Leucine administration increased the intracellular leucine concentration in muscle of control and septic rats to the same level. Plasma glutamine concentrations did not differ in control and septic rats under either basal conditions or 1 h after leucine (Fig. 6c). The intramuscular glutamine concentration was reduced ~50 % by sepsis in both saline- and leucine-treated groups (Fig. 6d).

Finally, as leucine is a known insulin secretagogue, the plasma insulin concentration was also assessed. The basal plasma insulin did not differ between control and septic rats (105 ± 11 vs. 92 ± 12 pmol/L, respectively; $P > 0.05$). Moreover, the plasma insulin concentration 1 h after oral leucine also did not differ between control and septic rats (112 ± 15 vs. 108 ± 14 pmol/L, respectively; $P > 0.05$).

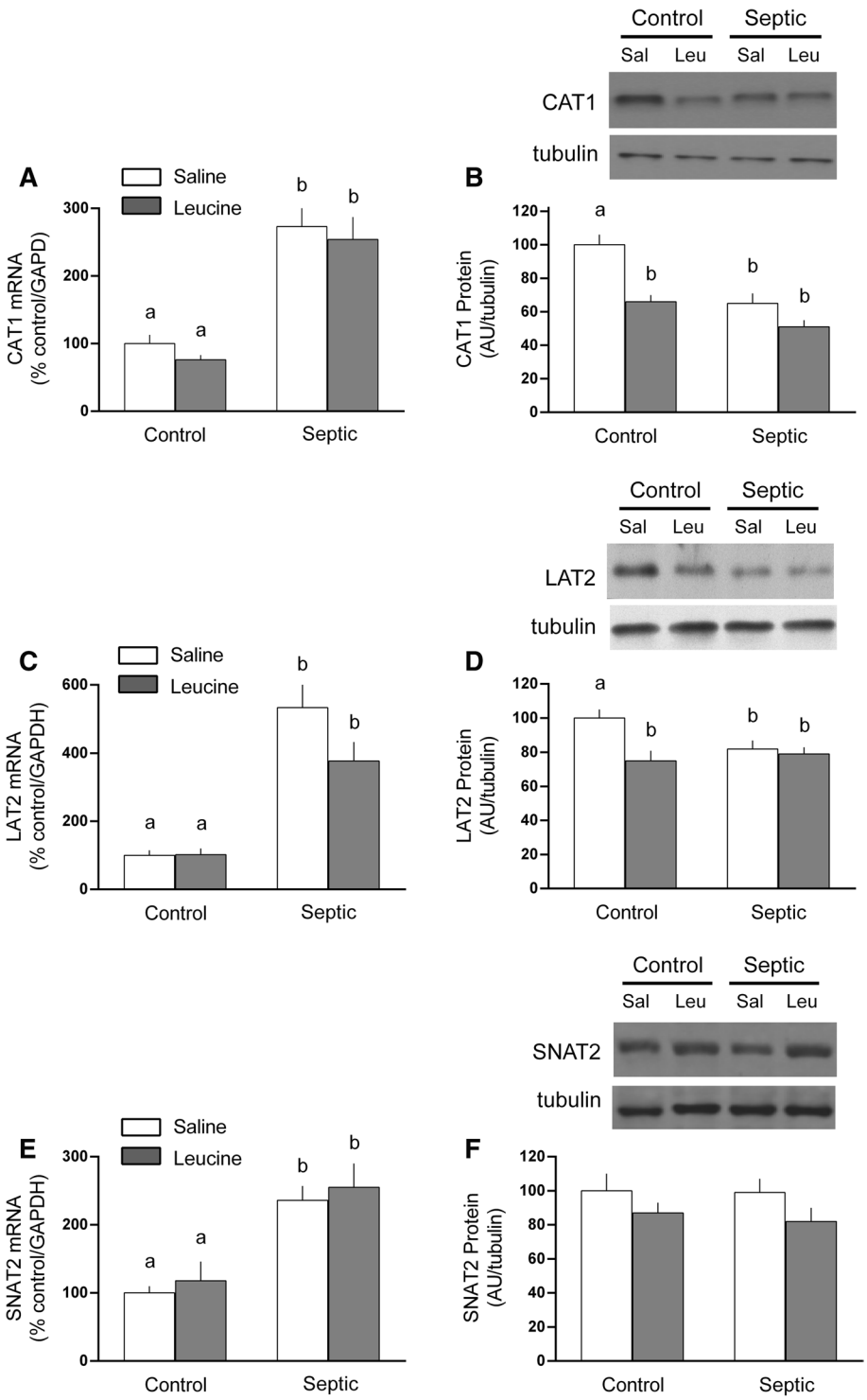

Fig. 2 Protein and mRNA content for amino acid transporters CAT1, LAT2 and SNAT2 in gastrocnemius from control and septic rats under basal conditions or 1 h after oral administration of leucine (Leu) or saline (Sal). For bar graphs, values are mean ± SEM; $n = 8$, 9, 9 and 9, respectively, for the four experimental groups. The mRNA content is expressed as percent of control normalized for GAPDH, where the value for the nonseptic control value was arbitrarily set at 100 %. Values having a different *superscript letter* (*a* vs. *b*) are statistically different ($P < 0.05$); values with the *same letter* are not significantly different. Representative Western blots are provided for each experimental group and for each amino acid transporter protein

Discussion

The present study investigated potential regulatory steps by which sepsis might impair leucine activation of mTOR-dependent muscle protein synthesis. As amino acid transporters facilitate the import of extracellular leucine into the cell as well as potentially function as nutrient sensors (Hatzoglou et al. 2004; Hundal and Taylor 2009; Ogmundsdottir et al. 2012), we first examined amino transporters implicated in leucine homeostasis. Although it does not directly transport leucine, CAT1 transports ornithine into the cell where it is metabolized to glutamine (Hatzoglou et al. 2004), which may then indirectly affect leucine transport via the LAT1 leucine/glutamine symporter

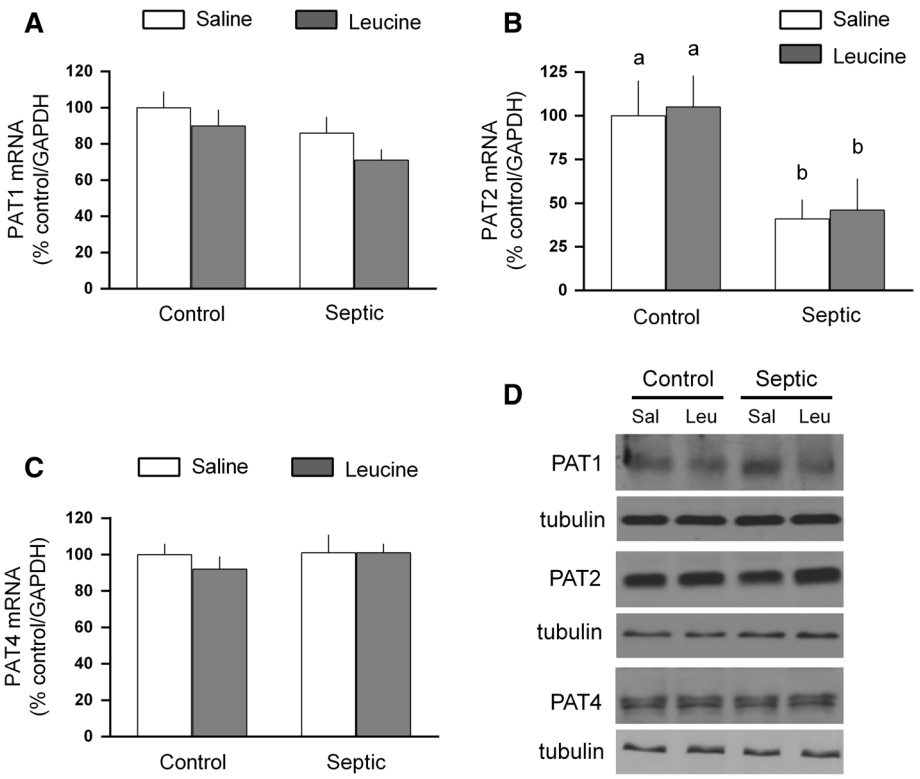

Fig. 3 Protein and mRNA content for proton-assisted amino acid transporter (PAT)-1, -2 and -4 in gastrocnemius from control and septic rats under basal conditions or 1 h after oral administration of leucine (Leu) or saline (Sal). **a–c**, *mRNA content* for each transporter was quantified and presented as a *bar graph*; data are expressed as percent of control normalized for GAPDH, where the value for the nonseptic control value was arbitrarily set at 100 %. For *bar graphs*, values are mean ± SEM; $n = 8$, 9, 9 and 9, respectively, for the four experimental groups. Values having a different *superscript letter* (*a* vs. *b*) are statistically different ($P < 0.05$); values with the *same letter* are not significantly different. **d**, representative Western blots are provided for each experimental group and for each protein; as no statistically significant sepsis or leucine effect was detected for PAT-1, -2 or -4 (data not shown) data were not presented as *bar graphs*

(Fig. 7). Increased CAT1 mRNA is seen in response to elevated glucocorticoids and amino acid starvation and results from increased transcription and mRNA stability (Liu and Hatzoglou 1998; Fernandez et al. 2002). However, in contrast to these conditions where CAT1 mRNA and protein were concomitantly increased, we detected a sepsis-induced decrease in CAT1 protein despite more than doubling of CAT1 mRNA expression. In general terms our data indicate that CAT1 translation is impaired, which is consistent with the sepsis-induced decrease in translational efficiency previously reported in skeletal muscle (Kazi et al. 2011; Lang et al. 2010) and which is mediated in part by increased systemic and local concentrations of proinflammatory cytokines and glucocorticoids (Lang and Frost 2006, 2007; Lang et al. 1996). A similar discordance between CAT1 transporter mRNA and protein content in muscle has been reported in the catabolic condition of extended bed rest (Drummond et al. 2012). At least for sepsis, this discrepancy between mRNA and protein may be due to the absence of an increase in eIF2α phosphorylation in muscle (Lang, unpublished observations), which was central to the increased translation of CAT1 in response to amino acid deprivation (Fernandez et al. 2002).

Transporters in the SLC38 family (System A) control influx of various small neutral amino acids, including glutamine, and comprise the various SNAT isoforms (Mackenzie and Erickson 2004). Of these, SNAT2 is the most widely expressed, is present in skeletal muscle, and regulated by growth factors, amino acid supply, and various stressors (Hyde et al. 2005, 2007). The SNAT symporters regulate the uphill flux of amino acids into the cell coupled with the inward movement of Na^+ down its electrochemical gradient. In turn, the intracellular glutamine can then be exported in exchange for leucine and other branched-chain amino acids via the System L transporters LAT1 and 2 (Hundal and Taylor 2009). Inhibition or knockdown of SNAT2 in cultured myotubes decreases intracellular amino acids, including leucine and impairs mTOR activity and protein synthesis (Evans et al. 2007). Our results indicate that while sepsis increased SNAT2 mRNA, the SNAT2 protein content in muscle did not differ between control and septic rats. These data are consistent with the

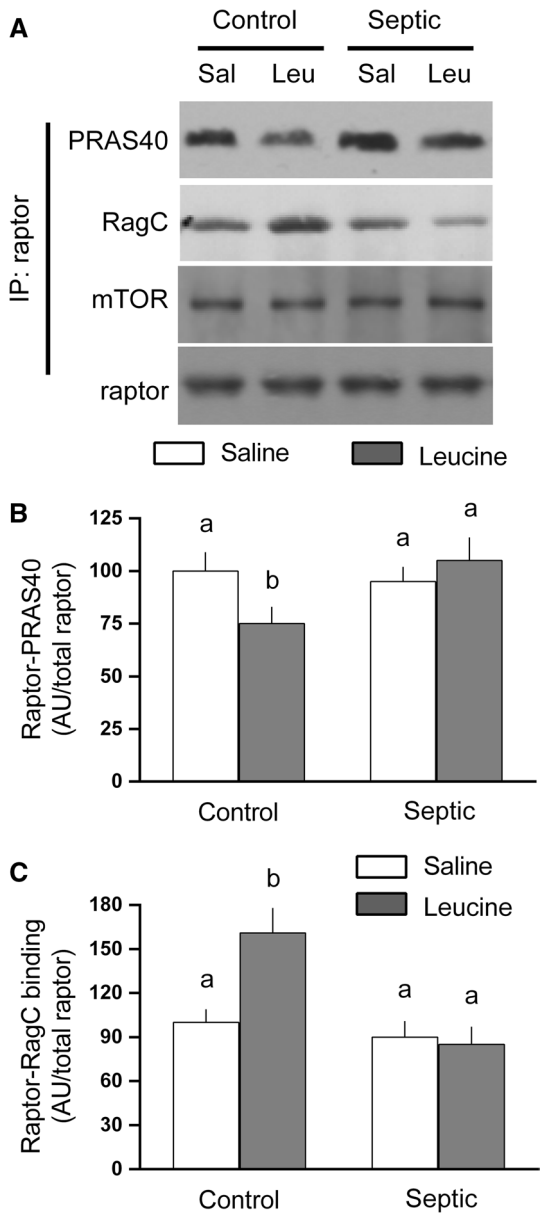

Fig. 4 Binding of raptor with PRAS40 and RagC in gastrocnemius from control and septic rats under basal conditions or 1 h after oral administration of leucine (Leu) or saline (Sal). For *bar graphs*, values are mean ± SEM; $n = 8$, 9, 9 and 9, respectively, for the four experimental groups. Values having a different *superscript letter* (*a* vs. *b*) are statistically different ($P < 0.05$); values with the *same letter* are not significantly different. Representative Western blots from the raptor immunoprecipitate (IP) are provided for each experimental group and for each protein

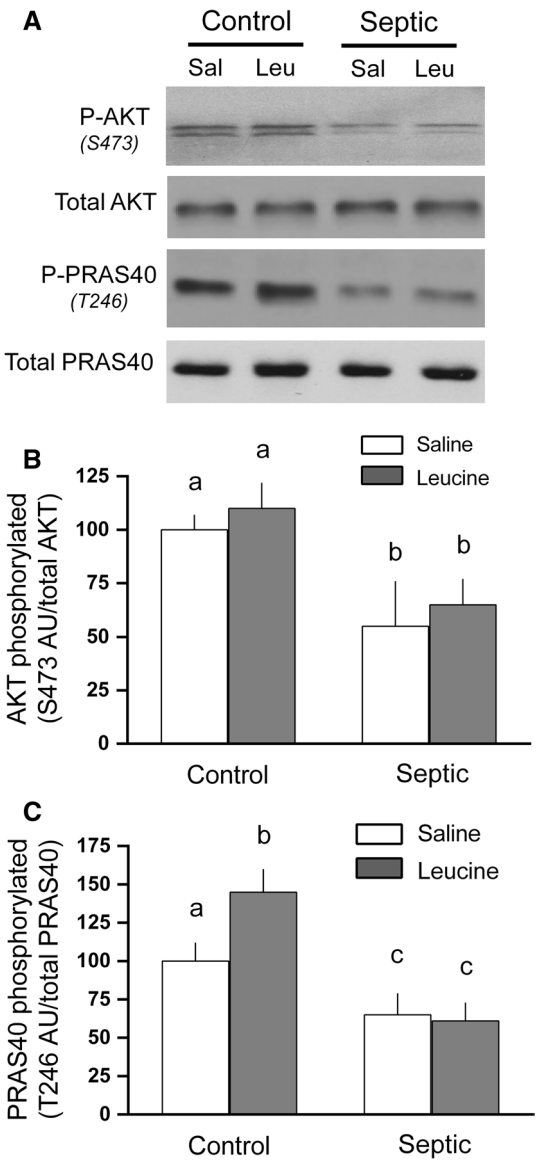

Fig. 5 Total and phosphorylation of AKT and PRAS40 in gastrocnemius from control and septic rats under basal conditions or 1 h after oral administration of leucine (Leu) or saline (Sal). For *bar graphs*, values are mean ± SEM; $n = 8$, 9, 9 and 9, respectively, for the four experimental groups. Values having a different *superscript letter* (*a* vs. *b* vs. *c*) are statistically different ($P < 0.05$); values with the *same letter* are not significantly different. Representative Western blots are provided for each experimental group and for each protein

lack of change SNAT2 seen in humans following extended bed rest which also produces muscle atrophy (Drummond et al. 2012). Additionally, the oral leucine load did not alter mRNA or protein content in control muscle for any of the amino acid transporters examined, confirming a previous report (Suryawan et al. 2013). Our data extend these findings to muscle from septic rats. Although this lack of effect for both control and septic muscle might be explained by the relatively short duration of leucine stimulation, other in vivo studies have reported increased expression of one or more transporter in a similar time frame (Drummond et al. 2010, 2012; Suryawan et al. 2013).

Our data indicate sepsis decreases the intracellular glutamine concentration, independent of a change in the circulating concentration of this amino acid, a finding which

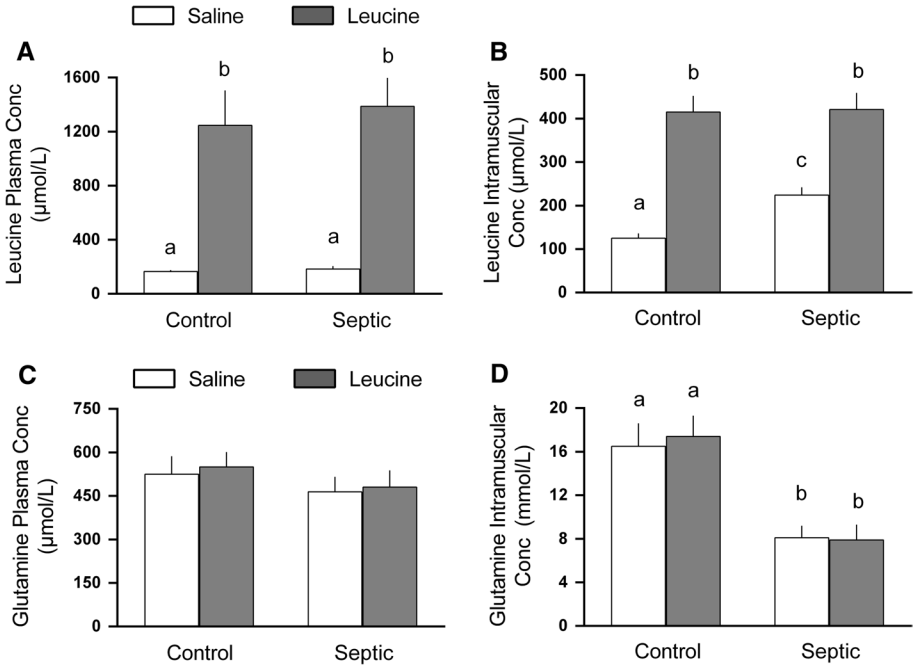

Fig. 6 Concentration of leucine and glutamine in plasma and gastrocnemius in control and septic rats under basal conditions or 1 h after oral administration of leucine (Leu) or saline (Sal). Values are mean ± SEM; $n = 7$ per group. Values having a different *superscript letter* (*a* vs. *b* vs. c) are statistically different ($P < 0.05$); values with the *same letter* are not significantly different

has been previously reported (Roth et al. 1982; Askanazi et al. 1980). This result is consistent with the above-mentioned sepsis-induced reduction in CAT1 protein which imports ornithine which can then be converted intracellularly to glutamine. In contrast, the sepsis-induced increase in intracellular leucine was paradoxically associated with a decrease in LAT2 protein. This discordance might be more due to the mismatch between the increased rate of leucine appearance from protein breakdown and the increased rate of leucine oxidation by muscle (Wolfe et al. 1989), than any change in plasma membrane amino acid transporter content. Furthermore, both the plasma and intracellular leucine concentrations were increased to a comparable magnitude in control and septic rats gavaged with leucine. While the leucine-induced increase in intracellular leucine has been reported after 1–3 h in normal subjects (Drummond et al. 2010), a comparable increase in septic muscle has not been previously reported. Hence, the diminished anabolic response to leucine in septic muscle appears independent of differences in either the circulating or intracellular leucine concentration, and cannot be explained by changes in the protein content for selected amino acid transporters.

The PATs are responsible for transport of small amino acids, and while they do not transport leucine per se, PATs have been reported to have characteristics of intracellular amino acid sensors (Goberdhan et al. 2009). Knockdown of PAT1 and PAT4 in cultured cells impairs mTOR kinase signaling and the normal rapid anabolic response of starved cells to amino acid refeeding (Heublein et al. 2010; Ogmundsdottir et al. 2012). Moreover, PAT1 is an mTOR binding partner and interacts in vivo with the RagGTPase at the surface of the late endosome/lysosome (Ogmundsdottir et al. 2012). There appear to be no reports of sepsis- or trauma-induced changes in this family of amino acid transporters. Our data indicate sepsis did not alter the mRNA content for PAT1 or PAT4, although a decreased PAT2 mRNA in muscle was noted. Moreover, the protein content for PAT1, -2 and -4 in muscle did not differ between control and septic rats. Further, there was no detectable change for either mRNA or protein for the PATs in response to acute leucine administration, and this finding is consistent with the previously reported inability of hyperaminoacidemia to alter PAT1 and PAT2 mRNA content in muscle (Suryawan et al. 2013). Collectively, these data suggest a difference in total PAT protein content is an unlikely mediator of the sepsis-induced decrease in muscle protein synthesis. However, we cannot exclude the possibility that sepsis alters the intracellular localization of one or more of the PATs and its subsequent association with the Rag proteins and mTORC1, as reported for the developmentally regulated decrease in mTOR activity (Suryawan et al. 2013).

The raptor protein functions as a scaffold recruiting select substrates to mTORC1 (Kim et al. 2002). As previously reported (Kazi et al. 2011), and confirmed herein, we did not detect a sepsis- and/or leucine-induced change in the total amount of mTOR or raptor, or the interaction of these two proteins which might be expected to alter kinase activity (Kim et al. 2002). Raptor also binds the Rag GTPases, which facilitates the recruitment of mTORC1 to the lysosome via a multi-protein complex identified as the Ragulator, which functions as a guanine nucleotide exchange factor activating the Rags and thus is central to

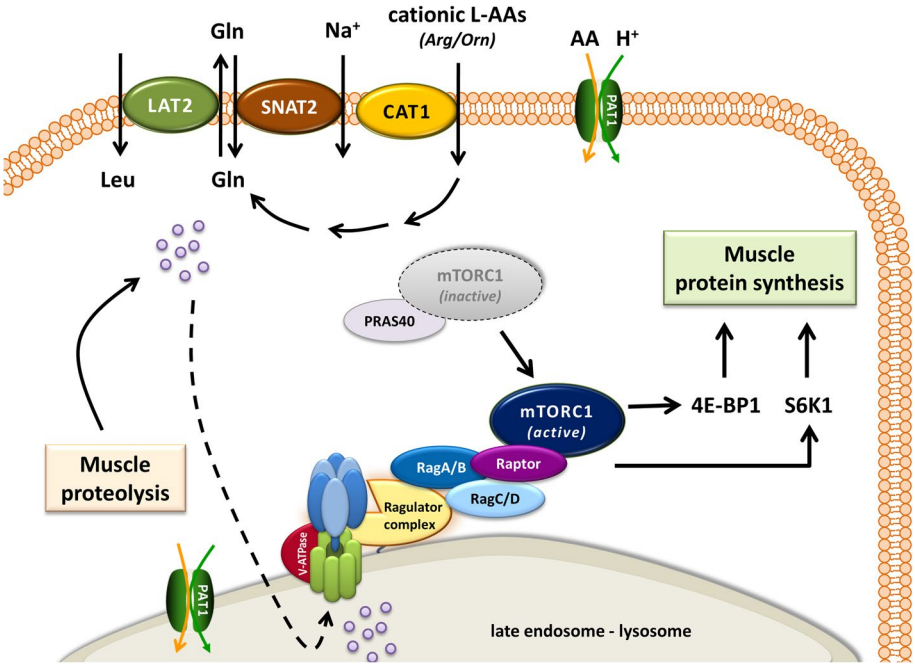

Fig. 7 Sepsis-induced leucine resistance in skeletal muscle. Model of muscle protein balance focusing on protein synthesis. Amino acids (AA) access the cell using numerous transporters on the cell membrane. Sodium-linked SNAT2 *(no change in sepsis)* mediates the uptake of neutral AAs such as glutamine (*Gln*), which can be exchanged with extracellular leucine (*Leu*) via LAT2 *(decreased in sepsis)*. Other AA transporters are also present on the cell membrane, such as CAT1 *(decreased in sepsis)* and the proton-assisted amino acid transports (PATs; no change in sepsis), the latter of which may also localized to the lysosome. Despite the sepsis-induced downregulation of various AA transporters, intracellular Leu is elevated, probably resulting from increased release of AAs via proteolysis (e.g., ubiquitin–proteasome and lysosomal/autophagy pathways). Leu is central for the stimulation of muscle protein synthesis and this is primarily mediated by the canonical mTOR signal pathway. It is posited that AAs enter the lysosome and signal via vacuolar (v)-ATPase which controls Ragulator–Rag association by triggering the GTP loading of Rags A/B. This active complex then binds directly to raptor which an integral part of mTOR complex (mTORC)-1 recruiting it to the lysosome. The enhanced mTOR kinase activity phosphorylates endogenous substrates, such as S6K1 and 4E-BP1 *(decreased in sepsis)*. Data herein show that Leu increases PRAS40 phosphorylation which likely leads to its dissociation from inactive mTORC1, thereby permitting raptor-mTOR binding to the active Ragulator–Rag complex. Sepsis prevents Leu-stimulated PRAS40 phosphorylation (mechanism unknown, but AKT independent) and limits the association of raptor with the Ragulator–Rag complex resulting in Leu resistance

amino acid sensing (Bar-Peled et al. 2012; Sancak et al. 2010). The present study indicated the total amounts of several mTORC1-interacting proteins (e.g., Rheb, LAMTOR1 [p18], LAMTOR2 [p14] and Rag A-D) within the whole tissue homogenate did not differ between control and septic rats and were not changed acutely by leucine. Other proteins (e.g., HBXIP and C7orf59) are present in the Ragulator (Bar-Peled et al. 2012; Sancak et al. 2010), but were not investigated. Furthermore, the amount of PRAS40 and RagC (e.g., negative and positive mTORC1 regulators, respectively) bound to the raptor immunoprecipitate did not differ between control and septic muscle in the basal condition. In contrast, it is noteworthy that the leucine-induced decrease in PRAS40·raptor binding and the increase in RagC·raptor binding seen in control muscle was not observed in septic rats. These results support previous studies in cultured cells and perfused liver, which reported similar changes in the association of PRAS40 and RagC with mTORC1 in response to insulin and nutrients (Dennis et al. 2011; Sancak et al. 2007) and extend them to intact skeletal muscle. Also, the inability of leucine to decrease PRAS40·raptor and increase RagC·raptor in septic rats is consistent with the observed leucine resistance in these animals. The leucine-induced decrease in PRAS40 binding to raptor in control rats is consistent with the concomitant increase in PRAS40 phosphorylation. While these data stress the potential importance of changes within mTORC1 related to the binding of PRAS40 and RagC to raptor as a mediator for the leucine resistance (Fig. 7), other potential regulators of the Rag GTPase, such as MAP4K3, v-ATPase, leucyl-tRNA synthetase, and SH3BP4 (Zoncu et al. 2011; Findlay et al. 2007; Han et al. 2012) remain to be elucidated.

Although observational, our results suggest the sepsis-induced leucine resistance observed in vivo in skeletal muscle occurs in the presence of elevated intracellular leucine which would appear to minimize the importance of the sepsis-induced decrease in CAT1 and LAT2 as a mechanism

for the leucine resistance. While the content of the various proteins within mTORC1 and the Ragulator were unchanged in sepsis, the normal leucine-induced decrease in raptor·PRAS40 and increase in raptor·RagC were absent in muscle from septic rats. These data suggest, but do not prove, a putative mechanism for the development of anabolic resistance in this catabolic condition. A better understanding of the molecular etiology for the sepsis-induced leucine resistance may lead to drug-based or nutraceutical interventions for this patient population, which would ameliorate the loss of LBM thereby improving recovery.

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Conflict of interest The authors state that they have no conflict of interest to declare.

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