Skeletal muscle protein balance in mTOR heterozygous mice in response to inflammation and leucine


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Lang CH, Frost RA, Bronson SK, Lynch CJ, Vary TC. Skeletal muscle protein balance in mTOR heterozygous mice in response to inflammation and leucine. Am J Physiol Endocrinol Metab 298: E1283–E1294, 2010. First published April 13, 2010; doi:10.1152/ajpendo.00676.2009.—Sepsis and lipopolysaccharide (LPS) may decrease skeletal muscle protein synthesis by impairing mTOR (mammalian target of rapamycin) activity. The role of mTOR in regulating muscle protein synthesis was assessed in wild-type (WT) and mTOR heterozygous (+/−) mice under basal conditions and in response to LPS and/or leucine stimulation. No difference in body weight of mTOR+/− mice was observed compared with WT mice; whereas whole body lean mass was reduced. Gastrocnemius weight was decreased in mTOR+/− mice, which was attributable to a reduced rate of basal protein synthesis. LPS decreased muscle protein synthesis in WT and mTOR+/− mice to the same extent. Reduced muscle protein synthesis in mTOR+/− mice under basal and LPS-stimulated conditions was associated with lower 4E-BP1 and S6K1 phosphorylation. LPS also decreased PRAS40 phosphorylation and increased phosphorylation of raptor and IRS-1 (Ser307) in WT and mTOR+/− mice. Muscle atrogin-1 and MuRF1 mRNA content was elevated in mTOR+/− mice under basal conditions, implying increased ubiquitin-proteasome-mediated proteolysis, but the LPS-induced increase in these atrogenes was comparable between groups. Plasma insulin and IGF-I as well as tissue expression of TNFα, IL-6, or NOS2 did not differ between WT and mTOR+/− mice. Finally, whereas LPS impaired the ability of leucine to stimulate muscle protein synthesis and 4E-BP1 phosphorylation in WT mice, this inflammatory state rendered mTOR+/− mice unresponsive. These data support the idea that the LPS-induced decrease in muscle protein synthesis produced by sepsis in the mature host is still unclear. As mice null for mTOR (whole-body) are embryonically lethal (20, 38), and mice with muscle-specific mTOR inactivation have a severe muscle pathology and die prematurely (43), we addressed this question by generating mice that are heterozygous for mTOR with an ~50% reduction in total mTOR protein in skeletal muscle and other tissues. Using these haploinsufficient mice and their wild-type (WT) littermates, we addressed the hypothesis that reduction of mTOR would decrease basal skeletal muscle protein synthesis and exaggerate the decrement in synthesis rates observed after LPS administration. Moreover, we anticipated that such mTOR knockdown in vivo would also produce a marked resistance to the anabolic effects of growth factor stimulation, supporting a causative role for deficient mTOR signaling in the LPS- and sepsis-induced decreases in muscle protein synthesis observed in vivo.

MATERIALS AND METHODS

Animals and experimental protocol. All mice were housed under specific pathogen-free conditions. Experimental mice were either mTOR heterozygous (mTOR+/−) or WT male littermates. Mice were housed under controlled environmental conditions and maintained on Teklad Global 2019 (Harlan Teklad, Boston, MA) and water ad libitum. All breeding and experimental protocols were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine and adhered to the National Institutes of Health (NIH) guidelines for the use of experimental animals.

Generation of FRAP1TMICLYN mice. The murine mTOR gene is located on chromosome 4 and consists of 58 exons spanning over 109 kilobases (kb) with a CDNA of 8.6 kb and 68 in-frame ATGs. A single point mutation at amino acid D2338 in exon 50 results in a kinase-dead protein. A gene targeting vector was created that introduced loxP recognition sites at either end of a 2.5-kb genomic region encompassing either lipopolysaccharide (LPS; endotoxin), a component of the outer cell wall of gram-negative bacteria, or tumor necrosis factor-α (TNFα), one of several inflammatory mediators released in response to bacterial infection and LPS (31, 32).

The ability of sepsis and LPS to impair muscle protein synthesis results, at least in part, from a decreased mTOR (mammalian target of rapamycin) kinase activity, as evidenced by the reduced phosphorylation of eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1) and ribosomal protein S6 kinase-1 (S6K1) (10, 33). Furthermore, a comparable reduction in protein synthesis and mTOR activity is observed under in vitro conditions in myocytes incubated with LPS and interferon-γ (IFNγ) or TNFα (19, 36). In general, mTOR senses and integrates inputs from nutrients, energy status, growth factors, and stress (13, 23) and is important for postnatal muscle growth, as evidenced by the severe atrophy observed in mice with muscle-specific inactivation of mTOR (43). However, whether alterations in mTOR are causally related to the reduced muscle protein synthesis produced by sepsis in the mature host is still unclear. As mice null for mTOR (whole-body) are embryonically lethal (20, 38), and mice with muscle-specific mTOR inactivation have a severe muscle pathology and die prematurely (43), we addressed this question by generating mice that are heterozygous for mTOR with an ~50% reduction in total mTOR protein in skeletal muscle and other tissues. Using these haploinsufficient mice and their wild-type (WT) littermates, we addressed the hypothesis that reduction of mTOR would decrease basal skeletal muscle protein synthesis and exaggerate the decrement in synthesis rates observed after LPS administration. Moreover, we anticipated that such mTOR knockdown in vivo would also produce a marked resistance to the anabolic effects of growth factor stimulation, supporting a causative role for deficient mTOR signaling in the LPS- and sepsis-induced decreases in muscle protein synthesis observed in vivo.

GRAM-NEGATIVE INFECTION causes loss of skeletal muscle protein and leads to the erosion of lean body mass (LBM) (5, 40, 50, 51, 55). This loss of muscle mass is clinically relevant, as it is associated with increased morbidity and mortality in sepsis and other catabolic conditions (2, 27). Sepsis decreases muscle mass by both increasing the rate of proteolysis and decreasing the rate of protein synthesis under basal conditions (5, 40, 50, 51). Moreover, sepsis also blunts the ability of select anabolic agents to stimulate protein synthesis and inhibit protein breakdown (14, 24, 28, 30). The presence of live bacteria is not a prerequisite for the dysregulation of muscle protein metabolism, as comparable changes are detected after the administration of
ing exons 49 and 50 of the murine mTOR locus (Fig. 1). The targeting vector also included a G418 resistance cassette followed by a third loxP recognition site immediately downstream of the latter loxP site. Additional sequence homology brought the 5′ arm to 5.5 kb and the 3′ arm to 4.1 kb. The targeting vector was then electroporated into C57BL/6J embryonic stem (ES) cells and resultant, correctly targeted, euploid ES cell clones were injected into blastocysts for creation of chimeric mice capable of transmitting the genetic change to the mouse germline.

**Generation of heterozygotes for disrupted mTOR gene.** Mice heterozygous for the recombinant FRAP (FK506-binding protein-12-rapamycin-associated protein)-1 allele (3 loxP sites containing the Neo expression cassette) were bred to mice hemizygous for a Cre recombinase transgene under the control of a cytomegalovirus minimal promoter that is expressed early in embryogenesis [B6.C-Tg(CMV-cre)1Cgn/J, 0060654, The Jackson Laboratory, Bar Harbor, ME]. The CMV-Cre transgene was introduced on the Balb/c background, partially backcrossed to C57BL/6J, and subsequently backcrossed an additional nine generations to C57BL/6J prior to intercross with the mTOR heterozygous mice. Complete resolution of the loxP sequences by Cre recombinase yields deletion of the G418 resistance cassette and the 2.5-kb region encompassing exons 49 and 50, including sequences critical for kinase activity and concomitantly causing a frameshift mutation (Fig. 1). The frame shift mutation was expected to result in an unstable message or an inactive and unstable mTOR peptide. Mice heterozygous for the null mTOR allele (1 loxP site) that did not carry the Cre transgene were further crossed to C57BL/6J to maintain the line. C57BL/6J stocks were imported from The Jackson Laboratory and bred no more than one generation at Penn State. In preliminary studies we genotyped 35 pups from six litters arising from the interbreeding of mice heterozygous for the mTOR-null allele. Of the progeny from heterozygous intercrosses, 74% were heterozygous for the null mTOR allele and 26% were homozygous for the WT allele. None of the offspring were homozygous null, although ~25% would have been expected in the absence of negative selection for this genotype. Chi-square analysis indicated that the probability of observing these genotypic ratios from a heterozygote intercross in the absence of embryonic lethality with the mTOR homozygous-null genotype is extremely low (P < 0.005), a conclusion consistent with previous independent reports (20, 38). Western blot analysis of gastrocnemius, heart, and liver from WT and mTOR−/− mice indicated an ~50–60% reduction in the amount of both total mTOR and phosphorylated protein (Fig. 1, inset, bottom). We used whole body mTOR heterozygous mice in this study instead of a muscle-specific mTOR knockout, because under in vivo conditions sepsis and LPS reduce mTOR phosphorylation by ~50% (29), and there are no naturally occurring physiological or catabolic conditions that are characterized by the complete loss of mTOR protein in skeletal muscle. Therefore, although the use of whole body mTOR heterozygous mice somewhat limits data interpretation, we believe this animal model represents a more physiological experimental paradigm.

**Genotyping assay.** Ear biopsies were obtained for genotyping. The presence of a disrupted mTOR allele DNA was determined by PCR amplification with the following primers: primer 1–5′ CCA CGC ATG GCC CAC TGT CTT T′3′ antisense; primer 4–5′ GCA TGG CGA GGA CAT GTC A′3′ antisense; and primer 5–5′ GTC CAC CAA CTC.

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**Fig. 1. Schematic representation of wild-type (WT) and recombinant (FRAP1TMICLYN) alleles of mTOR.** Inset, bottom: representative Western blot analysis of total and Ser2448-phosphorylated mammalian target of rapamycin (mTOR) in gastrocnemius, heart, and liver from WT and mTOR−/− mice. Also, total eIF4E is shown as a loading control.
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GGC CCT CAT T’3 sense. Products were separated on horizontal agarose gels for observation of products with the following sizes: 355 bp, indicating amplification with primers 4 and 5 and the presence of the WT allele (undisrupted, +), and 460 bp, indicating amplification with primers 1 and 5 and the presence of one loxP site (disrupted allele, −) (data not shown).

Indirect calorimetry, locomotive activity, and body composition. Prior to the quantifying of tissue protein synthesis (see below), indirect calorimetry was used to determine whole body oxygen consumption at 15-min intervals during both a complete 12:12-h light-dark cycle (Oxymax; Columbus Instruments, Columbus, OH) during the last week of the study. Spontaneous locomotive activity was measured using infrared technology (OPT-M3, Columbus Instruments), after a 24-h equilibration period, to determine horizontal activity (Xiot), ambulation (Xamb), and vertical activity (Ziot). Body fat and lean body mass (LBM) were measured using a 1H-NMR system (Bruker Minispec, LF90, Woodlands, TX), as previously reported by our laboratory (47).

Animal protocols and tissue protein synthesis. The in vivo rate of protein synthesis in various tissues was determined in WT and mTOR+/− mice under basal control conditions (e.g., saline injection) and 4 h after intraperitoneal injection of Exechirica coli LPS (Sigma, St. Louis, MO: 026:B6; 250 µg/mouse). The dose of LPS and the timing of the experiment were selected because previous studies indicated a significant reduction in muscle protein synthesis in rats (29, 31). Food was removed from all animals at the time of saline or LPS administration; therefore, protein synthesis determinations in this study are time-matched for those in the second study, which were performed on 4-h-fasted mice. Protein synthesis was determined using the flooding-dose technique described by Garlick et al. (21) and modified in our laboratory (56). Briefly, mice were injected intraperitoneally with 1-1H]Phenylalanine (Phe; 150 mM, 30 µCi/ml) or 1 ml/100 g body wt). A blood sample was collected for the measurement of phenylalanine concentration and radioactivity. The rate of protein synthesis (nmol Phe incorporated/h·mg protein) was calculated using the specific radioactivity of the plasma Phe as the precursor pool, exactly as previously described (50, 55, 56). Immediately following the removal of the blood, the gastrocnemius, liver, and heart were rapidly excised, frozen between liquid nitrogen-cooled aluminum blocks, weighed, and stored at −70°C.

In a separate experimental protocol, 4-h-fasted mice in all four experimental groups were administered an oral gavage of either leucine (1.35 g/kg body wt) or saline, and skeletal muscle was excised 30 min thereafter. The dose and timing of leucine administration were based on prior studies demonstrating maximal stimulation of muscle protein synthesis as well as phosphorylation of 4E-BP1 and S6K1 (28–30).

Ribo nuclease protection assays and northern blot analyses. Total RNA was extracted from tissues in a mixture of phenol and guanidine thiocyanate (TRI reagent; Molecular Research Center, Cincinnati, OH) using the manufacturer’s protocol. Riboprobe synthesis was from a multiprobe mouse cytokine template set (rCK-1; Pharmingen, San Diego, CA) using an in vitro transcription kit (Pharmingen). Primer sequences for other mRNAs, IGF-I, regulated in development (DIK2), were also determined. In general, blots were washed with TBS-T (1× TBS including 0.1% Tween 20) and 4E-BP1 and S6K1 were also determined. In general, blots were washed with TBS-T (1× TBS including 0.1% Tween 20) and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit) at room temperature. In addition, elf4E was immunoprecipitated from aliquots of supernatants using an anti-elf4E monoclonal antibody (Dr. J. Chambard, Hershey, PA), and the 4E-BP1-elf4E and elf4G-elf4E complexes were quantified exactly as described previously (30–32). Blots were incubated with enhanced chemiluminescence (ECL) reagents as per the manufacturer’s (Amersham) instructions, and dried blots were exposed to X-ray film for 1–30 min to achieve a signal within the linear range. After development, the film was scanned (Microtek ScanMaker IV) and analyzed using NIH Image 1.6 software.

Plasma hormones. The plasma concentrations of insulin and IGF-I were determined by ELISA (Linco Research, St. Charles, MO, and Immunodiagnostics Systems, Fountain Hills, AZ, respectively).

Statistical analysis. Data for each condition are summarized as means ± SE where the number of mice per treatment group is indicated in the legend to the figure or table. Unless otherwise indicated, statistical evaluation of the data was performed using analysis of variance with post hoc Student-Neuman-Keuls test when indicated. Differences were considered significant when P < 0.05.

RESULTS

Body composition, tissue weight, energy expenditure, and activity. There was no significant difference in the total body weight between WT and mTOR+/− mice (Fig. 2). However, a small albeit statistically significant decrease in LBM was detected by 1H-NMR spectroscopy in mTOR+/− mice compared with time-matched WT control mice. This reduction in LBM was present whether data were expressed in absolute units (e.g., grams of muscle) or normalized to total body weight (Fig. 2). These changes were consistent with the reduction in the wet weight of the gastrocnemius and heart observed in heterozygous mice. In contrast, the absolute and relative percentages of fat mass tended to be increased in mTOR+/− mice. There was no difference in liver weight between the two groups of mice (Fig. 2). The above-mentioned changes in body composition were independent of food consumption between groups, which was not different between WT (4.9 ± 0.3 g/day) and mTOR+/− (4.9 ± 0.2 g/day) mice during the final 2 wk of the experimental protocol.

To determine whether the changes in body composition were due to changes in whole body energy balance, indirect calorimetry was performed. Oxygen consumption (ml·h−1·kg body wt−1) did not differ between WT and mTOR+/− mice in either the light (2.955 ± 0.185 and 2.861 ± 0.101, respectively, P > 0.05) or the dark phase (3.328 ± 0.97 and 3.276 ± 0.14, respectively, P > 0.05). Similarly, spontaneous locomotor activity (Xtotal, Xambulatory, and Ztotal) also did not differ between WT (29.410 ± 3.951, 14.753 ± 2.348, and 1.714 ± 3.20 counts/24 h, respec-
tively) and mTOR+/− (29,988 ± 1,970, 14,890 ± 2,197, and 1,979 ± 363 counts/24 h, respectively, P > 0.05) mice.

Tissue protein synthesis. The global rate of protein synthesis in gastrocnemius was decreased 20% in mTOR+/− mice compared with WT control values (Fig. 3, top). LPS acutely reduced muscle protein synthesis by 38% in WT mice and by 32% in mTOR+/− mice. In contradistinction, there was no difference in protein synthesis in heart and liver under basal conditions between WT and mTOR+/− mice, and LPS acutely increased protein synthesis in these two tissues to the same extent in both WT and mTOR+/− mice (Fig. 3, middle and bottom).

Alterations in mTOR kinase activity produce proportional changes in the phosphorylation of 4E-BP1, which in turn alters the distribution of eIF4E between active and inactive protein complexes and impacts cap-dependent translation (46). As illustrated in Fig. 4, under control conditions there was no difference in the amount of the hyperphosphorylated γ-isofrom or total amount of 4E-BP1 in skeletal muscle of mTOR+/− mice compared with WT control values. Despite the lack of change in γ-phosphorylated 4E-BP1, there was a small (30%), but statistically significant, increase in the amount of eIF4E bound to 4E-BP1 (e.g., inactivate eIF4F complex) in mTOR+/− mice. Conversely, the extent of eIF4E binding to eIF4G (e.g., active eIF4F complex) tended to be increased in mTOR+/− mice under control conditions, but this difference did not achieve statistical significance. Injection of LPS into WT mice decreased 4E-BP1 phosphorylation (50%), decreased eIF4E-eIF4G association (80%), and increased eIF4E-4E-BP1 association (85%) compared with saline-treated control values (Fig. 4). There was no difference in the LPS-induced changes in 4E-BP1 phosphorylation and the distribution of eIF4E between WT and mTOR knockdown mice. None of the above-men-
bands, indicating that the relative phosphorylation of S6K1 (Fig. 5). LPS injection markedly increased the mobility of the under basal conditions, which was not statistically different when [3H]phenylalanine (Phe) was injected ip and radiolabel incorporation into control conditions and 4 h after injection of LPS or saline vehicle (control).

When S6K1 is subjected to SDS-PAGE, it resolves into multiple bands with different electrophoretic mobilities depending on the extent of phosphorylation at various Ser/Thr sites. The most slowly migrating forms represent the heavily phosphorylated and thus the more active form of the kinase. This method was used to assess S6K1 phosphorylation levels because in the fasted state there is little Thr389-phosphorylated Akt detected in skeletal muscle (unpublished observation and Ref. 47). There was constitutive S6K1 phosphorylation in skeletal muscle from WT and mTOR+/− mice under basal conditions, which was not statistically different when compared with WT mice (Fig. 5).

The mTOR complex I (mTORC1) is composed in part by mTOR, GβL, raptor, and PRAS40 (13). These latter two components are phosphoproteins that affect mTOR kinase activity. Under basal control conditions, there was no difference in the amount of phosphorylated or total raptor and PRAS40 or total GβL between WT and mTOR+/− mice (Fig. 6). However, LPS increased raptor phosphorylation approximately twofold and reciprocally decreased PRAS40 phosphorylation by 50–55% in both WT and mTOR+/− mice (Fig. 6).

There was no difference in Thr308- or Ser473-phosphorylated Akt between WT and mTOR+/− mice under basal conditions (Fig. 7). In response to LPS, the amount of Thr308-phosphorylated Akt was reduced 45–55% in both WT and mTOR+/− mice, whereas the extent of Ser473-phosphorylated Akt did not differ between groups. Injection of LPS increased IRS-1 Ser307 phosphorylation twofold in both WT and mTOR+/− mice. Under basal conditions, the extent of Ser307-phosphorylated IRS-1 in the mTOR+/− mice was intermediate between basal values from WT mice and the increases produced by LPS (Fig. 7).

Although selective cellular stresses have also been reported to inhibit both basal and insulin-induced activation of mTOR activity via p53-dependent upregulation of Sestrin2 (Sesn2) (6), no change in Sesn2 content was observed in muscle from mTOR+/− and WT mice or in response to LPS (data not shown). Likewise, while activation of AMPK decreases mTOR kinase activity and muscle protein synthesis (41), total and Thr172-phosphorylated AMPK in muscle also did not differ among the four experimental groups (data not shown).

We also performed Western blot analysis on heart from all groups and detected no consistent effect of either LPS or heterozygous mTOR gene deletion on total and phosphorylated 4E-BP1, S6K1, raptor, PRAS40, or IRS-1 phosphorylation. Also, there was no significant change in the amount of eIF4E-eIF4BP1 or eIF4-eIF4G complex formation among the four experimental groups for heart (data not shown). Livers from mice in the various groups were not analyzed further.

Surrogate markers of muscle protein balance. The mRNA content of both atrogin-1 and MuRF1 was increased 70–80% in gastrocnemius from mTOR+/− mice compared with time-matched WT controls (Fig. 8). Although LPS increased MuRF1 mRNA in both WT and mTOR+/− mice to a comparable extent, LPS increased atrogin-1 mRNA only in WT control mice. The muscle mRNA expression of REDD1 was also increased 75% in mTOR+/− mice, and LPS increased the REDD1 mRNA approximately eightfold in both WT and mTOR+/− mice compared with their respective basal values. Although there was no significant difference in the IGF-I mRNA content of gastrocnemius in WT and mTOR+/− mice under basal conditions, LPS decreased muscle IGF-I mRNA more than 50% in the mTOR+/− mice but not WT mice (Fig. 8).

Muscle cytokines. Muscle is a component of the innate immune system and synthesizes and secretes a number of inflammatory cytokines that can influence tissue metabolism (17). In this regard, we quantified the mRNA content for Il1β, which is upregulated by LPS, IL-1, IL-6, and TNFα (53) in...
addition to more traditional inflammatory cytokines. There was no difference in the muscle mRNA content for various inflammatory mediators between WT and mTOR<sup>+/−</sup> mice under basal control conditions and after injection of LPS. Inset: representative Western blots of total mTOR and total 4E-BP1 in muscle homogenate where α-, β-, and γ-isoforms of 4E-BP1 are identified. When all 3 isoforms were quantified and summed, there were no differences for total 4E-BP1 among the 4 groups. In addition, eIF4E was immunoprecipitated (IP), and the amount of eIF4G and 4E-BP1 bound to eIF4E was assessed by immunoblotting (IB). For eIF4E-eIF4G and eIF4E-4EBP1 complexes, the amount of eIF4E in the IP did not differ among groups. Values are means ± SE; n = 9, 12, 12, and 12, respectively. Values with different superscript letters a, b, and c are statistically different (P < 0.05) from each other; values with the same letter are not statistically different.

Plasma insulin and IGF-I. The plasma concentrations of insulin and IGF-I are proportional to changes in protein synthesis and inversely related to protein breakdown in skeletal muscle. Under basal conditions, there was no significant difference for either insulin or IGF-I between WT and mTOR<sup>+/−</sup> mice (Fig. 9). The injection of LPS increased the insulin concentration ~2.5-fold in both WT and mTOR<sup>+/−</sup> mice. The plasma IGF-I concentration, however, was reduced in mTOR<sup>+/−</sup> mice only in response to LPS but not in similarly treated WT mice.

Acute leucine stimulation. In WT control mice, leucine acutely increased muscle protein synthesis compared with WT mice gavaged with saline (Fig. 10, top). The leucine-induced increment in protein synthesis was ~50% smaller in mTOR<sup>+/−</sup> mice under control (e.g., no LPS) conditions, and a similar decrement was detected in WT mice injected with LPS. Finally, the ability of leucine to increase muscle protein synthesis was completely lost in mTOR<sup>+/−</sup> mice treated with LPS. Statistical analysis was not performed on WT and mTOR<sup>+/−</sup> mice with or without LPS injection and receiving an oral gavage of saline (open bars) because these animals are largely equivalent to the four group of animals included in the first series of experiments (oral gavage vs. ip injection of saline) and exhibited qualitatively similar changes as reported earlier.

In addition, the phosphorylation of 4E-BP1 and ribosomal protein S6 in skeletal muscle were assessed (Fig. 10, middle and bottom, respectively). In the control condition, the ability of leucine to increase phosphorylation of 4E-BP1 was comparable between WT and mTOR<sup>+/−</sup> mice. LPS administration to WT mice decreased leucine-induced 4E-BP1 phosphorylation more than 50%. Furthermore, LPS produced an exaggerated leucine resistance in muscle of mTOR<sup>+/−</sup> mice, as evidenced by the 80% reduction in 4E-BP1 phosphorylation in response to amino acid stimulation. Leucine-induced changes in Ser<sup>240/245</sup>-phosphorylated S6 were comparable to the above-mentioned leucine-induced changes in 4E-BP1 phosphorylation. In our study, the phosphorylation of S6 was assessed instead of S6K1 because constitutive Thr<sup>389</sup>-phosphorylated S6K1 is low and difficult to reliably detect in muscle from fasted WT mice. Hence, Ser<sup>240/245</sup>-phosphorylated S6 was determined as phosphorylation of these residues represents S6K1 activity (44, 47).
Other end points that were assessed in WT and mTOR<sup>+/−</sup> mice under basal control conditions and after injection of LPS. Top: arrows indicate different phosphorylated forms of S6K1. There was no statistical difference in phosphorylated S6 protein among the 4 groups (1.00 ± 0.09, 0.98 ± 0.08, 0.95 ± 0.07, and 0.94 ± 0.08 AU/total S6 protein) or for total S6 protein (data not shown). Values are means ± SE; n = 9, 12, 12, and 12, respectively. Values with different superscript letters a,b, and c are statistically different (P < 0.05) from each other; values with the same letter are not statistically different.

**DISCUSSION**

*Alterations in the basal metabolism in mTOR<sup>+/−</sup> mice.* Herein we present novel data related to muscle protein balance in mTOR<sup>+/−</sup> mice in the basal condition and their in vivo response to both catabolic and anabolic stimuli. While there was no discernable difference in total body weight between WT and heterozygous mice, confirming earlier reports (20, 38), the mTOR<sup>+/−</sup> mice did have a previously unreported decrease in LBM. The loss of LBM was attributable to a reduction in both skeletal and cardiac mass and was independent of a difference in caloric intake. An atrophic response for skeletal muscle

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**Fig. 5.** Phosphorylation of S6K1 and ribosomal protein (rp)S6 in skeletal muscle from WT and mTOR<sup>+/−</sup> mice under basal control conditions and after injection of LPS. Top: arrows indicate different phosphorylated forms of S6K1. There was no statistical difference in phosphorylated S6 protein among the 4 groups (1.00 ± 0.09, 0.98 ± 0.08, 0.95 ± 0.07, and 0.94 ± 0.08 AU/total S6 protein) or for total S6 protein (data not shown). Values are means ± SE; n = 9, 12, 12, and 12, respectively. Values with different superscript letters a,b, and c are statistically different (P < 0.05) from each other; values with the same letter are not statistically different.

**Fig. 6.** Phosphorylation of raptor and PRAS40 in skeletal muscle from WT and mTOR<sup>+/−</sup> mice under basal control conditions and after injection of LPS. There was no statistical difference in total raptor, PRAS40, or GβL among any of the groups (see top inset for representative data). Values are means ± SE; n = 9, 12, 12, and 12, respectively. Values with different superscript letters a,b, and c are statistically different (P < 0.05) from each other; values with the same letter are not statistically different.
muscle is also observed in S6K1-deficient mice, but in this model the decrease in muscle mass was proportional to the smaller body weight (48). No such decrease in the mass of either the liver or adipose tissue was detected in mTOR−/− mice. The skeletal muscle atrophy observed in mTOR−/− mice resulted, at least in part, from a decreased rate of basal protein synthesis, whereas the cardiac atrophy was independent of such a reduction. Likewise, an ~50% reduction in total protein synthesis rate in liver. This latter observation is consistent with that of Reiter et al. (42), in which global hepatic protein synthesis was not altered by in vivo administration of the mTOR inhibitor rapamycin. The reason for this relatively tissue-specific decrease in protein synthesis in mTOR−/− mice is unclear but may be related to the relative abundance of mTOR, which is substantially greater in heart and liver compared with skeletal muscle (unpublished observation).

The decreased basal muscle protein synthesis in mTOR−/− mice was not associated with a significant change in either 4E-BP1 or S6K1 phosphorylation, and these findings are in contrast to the impaired phosphorylation of these two proteins in mice harboring a muscle-specific deletion of raptor in skeletal muscle (3). It seems likely that the magnitude of the difference between the present study and that of Bentzinger et al. (3) relates to the partial reduction of mTOR vs. complete deletion of raptor in skeletal muscle. Such speculation is consistent with data from mice with muscle-specific inactivation of mTOR, which demonstrate severe muscle atrophy (43). Regardless, the increased amount of the inactive 4EBP1·eIF4E complex in muscle of mTOR−/− mice is internally consistent with the reduction in protein synthesis. Raptor, GβL, and PRAS40 are all components of the TORC1, which controls mTOR kinase activity (13, 22, 23, 25), but we detected no difference in either the total amount or the extent of phosphorylation of these proteins in skeletal muscle of mTOR−/− mice under basal conditions. These data suggest a limited role for changes in these particular proteins in regulating mTOR activity and protein synthesis in the mTOR−/− mice, although we acknowledge that the association of raptor and PRAS40 with mTOR is also of great importance in regulating translational activity and was not investigated in the current study.

Skeletal muscle atrophy in mTOR−/− mice may also result from an increased protein degradation. The regulation of muscle proteolysis is multifaceted, involving lysosomal, calcium-dependent, and ubiquitin (Ub)-proteasome-dependent mechanisms (35). Gastrocnemius from the heterozyous mice had nearly twofold increases in the mRNA content for the E3 ligases atrogin-1 and MuRF1, proteins believed to be important regulators of Ub-proteasome-dependent proteolysis (4, 35). These results appear to differ from those obtained in muscle-specific raptor knockout mice, which demonstrated a decrease in basal MuRF1 and atrogin-1 mRNA (3). The forkhead box O (FoxO) transcription factor signaling pathway is a primary regulator of atrogene expression. Decreased activation of Akt might be expected to reduce FoxO3 phosphorylation and increase transcription of atrogin-1 and MuRF1 (49). However, the extent of Thr308- and Ser473-phosphorylated Akt did not differ between WT and mTOR−/− mice in the basal condition. Hence, if the FoxO pathway regulates atrogene expression in mTOR−/− mice, it must do so in an Akt-independent manner. Alternatively, the nuclear factor (NF)-κB

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pathway also regulates atrogene expression. For example, muscle-specific transgenic expression of activated IκB kinase-β (MIKK) leads to muscle atrophy that is in part mediated via activation of MuRF1 (7). Moreover, the increased MuRF1 mRNA content in muscle of these transgenic mice occurred in the absence of a concomitant increase in muscle inflammatory cytokines. Furthermore, recently, Tong et al. (52) have reported that the mTOR inhibitor rapamycin increased atrogene protein expression in C2C12 myotubes. The involvement of these or other pathways in regulating atrogene expression in the mTOR mice requires additional study. While increased atrogene mRNA is not a direct measure of muscle proteolysis, the coordinated upregulation of both atrogin-1 and MuRF1 is supportive of such a mechanism in the mTOR mice under basal conditions. These changes in muscle protein metabolism cannot be ascribed to differences in either the circulating insulin concentration or the amount of IGF-I in the blood or muscle per se between groups under basal conditions. Furthermore, there was no evidence of inflammation in muscle from mTOR mice that would explain the basal catabolic condition.

**Response of mTOR mice to a catabolic stimulus.** A decrease in muscle protein synthesis is observed acutely after LPS and chronically after bacterial infection, and such changes are strongly associated with impaired mTOR kinase activity (24, 26, 28, 29, 31, 50, 55). Our current data in WT mice confirm previous observations in rats and neonatal pigs indicating a concomitant reduction in both 4E-BP1 and S6K1 phosphorylation as well as a redistribution of eIF4E from the active eIF4E-eIF4G complex to the inactive eIF4E-eIF4BP1 complex. Furthermore, in muscle of...
WT mice, LPS also increased Ser792-phosphorylated raptor and decreased Thr246-phosphorylated PRAS40. Raptor is a scaffold protein that recruits other substrates to mTORC1 and appears necessary for optimal kinase activity and the phosphorylation of 4E-BP1 (13). Phosphorylation of raptor in an AMPK-dependent manner appears to recruit the 14-3-3 protein leading to inhibition of mTOR kinase activity (23). Although a comparable increase in raptor phosphorylation is also observed in rats after AMPK activation by AICAR (41), we did not detect an increase in AMPK activity (i.e., AMPK or ACC phosphorylation) in muscle 4 h after LPS administration. This lack of AMPK activation in muscle by LPS is consistent with reports in humans (16). Conversely, Thr246 phosphorylation of PRAS40 by Akt (e.g., in response to insulin) appears to release the normal inhibitory effect of PRAS40 on mTOR kinase activity (45). Hence, the LPS-induced decrease in PRAS40 phosphorylation is consistent with that reported after AICAR (41) as well as the reduction in Akt Thr308 phosphorylation in the present study. The ability of LPS to decrease Akt phosphorylation was associated with enhanced Ser607 phosphorylation of IRS-1, which is increased in other inflammatory conditions and impairs growth factor signaling in muscle (12). Similar bidirectional changes in raptor and PRAS40 phosphorylation were observed in skeletal muscle of WT and mTOR+/− mice under basal conditions and 4 h after injection of LPS. Plasma concentrations of these anabolic hormones were determined by ELISA. Values are means ± SE; n = 9, 12, 12, and 12, respectively. Values with different superscript letters a,b, and c are statistically different (P < 0.05) from each other; values with the same letter are not statistically different.

Fig. 9. Plasma concentration of insulin and IGF-I in WT and mTOR+/− mice under basal conditions and 4 h after injection of LPS. Plasma concentrations of these anabolic hormones were determined by ELISA. Values are means ± SE; n = 9, 12, 12, and 12, respectively. Values with different superscript letters a,b, and c are statistically different (P < 0.05) from each other; values with the same letter are not statistically different.

Fig. 10. Muscle protein synthesis and phosphorylation of 4E-BP1 and S6 in skeletal muscle of WT and mTOR+/− mice in response to in vivo leucine stimulation. Mice were orally gavaged with a maximally stimulating dose of leucine or an equal volume of saline, and gastrocnemius was removed 30 min thereafter. Values from WT mice control mice gavaged with saline were arbitrarily set at 1.0 AU. Values are means ± SE; n = 10, 12, 14, 9, 11, 12, and 12, respectively. *P < 0.05 vs. saline control values from the same treatment group, by Student’s t-test. Values with different superscript letters a, b, and c are statistically different (P < 0.05) from each other, by ANOVA and Student-Neuman-Keuls test.
phosphorylation have been reported in C2C12 myocytes cultured with a combination of LPS and interferon-γ and which are prevented by inhibition of NOS2 activity and the production of NO (19). It is noteworthy that all of the above-mentioned LPS-induced changes in the phosphorylation of proteins controlling mTOR activity in muscle are comparable in WT and mTOR+/− mice and are consistent with the similar LPS-induced decrease in muscle protein synthesis. The similar LPS-induced decrement in muscle protein synthesis was somewhat unanticipated, given that the drop in IGF-I in blood and muscle was greater in mTOR+/− vs. WT mice.

A number of diverse atrophic stimuli increase the gene expression of the muscle-specific E3 ligases atrogin-1 and MuRF1, and mice lacking these proteins are largely resistant to muscle denervation and TNF-induced muscle wasting (1, 4). Sepsis and LPS increase atrogin-1 and MuRF1 mRNA content in skeletal muscle in a dose- and time-dependent manner (11, 18, 57), and this elevation is regarded generally (39), but not universally (15), as an important contributor to the atrophic response. Furthermore, this increased gene expression is consistent with the direct measurement of muscle proteolysis that has been consistently reported to be increased by LPS and sepsis (8, 15). In the current study, LPS upregulated atrogin expression, and the magnitude of this response was comparable between WT and mTOR+/− mice. These results are consistent with the similar elevation in atrogin-1 and MuRF1 expression in WT and S6K1/S6K2 double-knockout mice in response to the catabolic conditions imposed by either starvation or denervation (37).

Acute leucine stimulation. In the final experimental series, WT and mTOR+/− mice were orally administered the branched-chain amino acid leucine and the acute anabolic response was assessed directly by measuring in vivo protein synthesis and indirectly by determining 4E-BP1 and S6K1 phosphorylation. Compared with their respective time-matched control value (Fig. 10, open bars), three of the four groups exhibited a leucine-induced increase in muscle protein synthesis. This anabolic response was greatest in WT control mice and was reduced in both mTOR+/− mice under basal conditions and WT mice injected with LPS. No leucine-induced increase in muscle protein synthesis was detected in mTOR+/− mice injected with LPS. We also determined whether changes in global protein synthesis correlated with changes in the phosphorylation state of 4E-BP1 and ribosomal protein S6. Leucine resistance, as evidenced by either 4E-BP1 or S6 phosphorylation, was not detected in mTOR+/− mice under basal conditions; however, LPS administration to WT mice resulted in a reduced level of 4E-BP1 and S6 phosphorylation in response to leucine. Finally, mTOR+/− mice given LPS were leucine unresponsive, showing no increase in 4E-BP1 of S6 phosphorylation in response to stimulation by this amino acid. Collectively, these observations are consistent with those findings from mouse embryonic fibroblasts prepared from mTOR+/− mice that had reduced Thr1899 S6K1 phosphorylation in response to serum stimulation (20). Moreover, our data suggest that diminished mTOR signaling predominates over the stimulatory effects of leucine.

In summary, mTOR+/− mice exhibit decreased muscle protein synthesis and increased proteolysis under basal conditions that were independent of alterations in muscle inflammatory cytokines and circulating growth factors. Whereas the protein catabolic responses of mTOR+/− and WT mice to LPS were comparable, the ability of the heterozygous mice to respond to the anabolic nutrient leucine was diminished under basal conditions and further worsened in response to LPS. These data support the contention that inflammation-induced reductions in mTOR activity play a central role in regulating skeletal muscle mass under basal conditions and especially in response to anabolic stimulation.

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DISCLOSURES

No conflicts of interest are reported by the authors.

REFERENCES


