Development Aggravates the Severity of Skeletal Muscle Catabolism Induced by Endotoxemia in Neonatal Pigs

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Short title: Development and endotoxin-induced muscle catabolism

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ABSTRACT

Accretion rates of muscle protein are elevated in normal neonates but this anabolic drive decreases with maturation. As this change occurs, it is not known whether development also influences muscle protein catabolism induced by sepsis. We hypothesize that protein degradation in skeletal muscle induced by endotoxemia becomes more severe as the neonate develops. Fasted 7- and 26-d-old pigs were infused for 8 h with LPS (lipopolysaccharide, 0 and 10 µg•kg⁻¹•h⁻¹) while plasma amino acids (AA), 3-methylhistidine (3-MH), and α-actin concentrations and muscle protein degradation signal activation were determined (n=5-7/group/age). Plasma full length α-actin was greater in 7- than 26-d-old pigs suggesting a higher baseline protein turnover in neonatal pigs. LPS increased plasma total AA, 3-MH, and full length and cleaved α-actin in 26- than in 7-d-old pigs. In muscle of both age groups, LPS increased AMPK and NFκB phosphorylation, the abundances of activated caspase 3 and E-3 ligases, MuRF1 and atrogin1, and the abundance of cleaved α-actin suggesting activation of muscle proteolysis by endotoxin in muscle. LPS decreased Fox01 and -4 phosphorylation and increased pro-caspase 3 abundance in muscle of 26-d-old pigs despite lack of effect of LPS on PKB phosphorylation. The results suggest that skeletal muscle in healthy neonatal pigs maintains high baseline degradation signal activation that cannot be enhanced by endotoxin, but as maturation advances, the effect of LPS on muscle protein catabolism manifests its severity.
Key words: AMPK, plasma α-actin, muscle wasting, catabolism.

Abbreviations

3-MH: 3-methylhistidine

AMPK: 5′ AMP-activated protein kinase

EAA: essential amino acids

LPS: lipopolysaccharide, endotoxin

MuRF1: muscle RING finger 1

NEAA: non-essential amino acids

NFκB: Nuclear factor κ B

PKB: protein kinase B
INTRODUCTION

Skeletal muscle catabolism is a crucial feature in the metabolic dysregulation that accompanies systemic inflammation and sepsis (2; 10; 32). During sepsis, increased amino acid (AA) flux from skeletal muscle are needed to provide substrate for an overall increase in whole body protein synthesis necessary to sustain the systemic inflammatory response (11). In skeletal muscle, sepsis and inflammation alter the molecular pathways that regulate the maintenance of muscle mass, by two mechanisms: 1] By diminishing the response to hormones and nutrients (e.g., insulin and AA) that stimulate protein deposition (12; 15); and 2] By decreasing baseline rates of protein synthesis and enhancing protein degradation to allow an AA flux to stream into the systemic circulation (10; 43). Thus, similar to cachexia and in contrast to starvation, the loss of muscle associated with sepsis is unresponsive to exogenous nutrient support (7; 20). The sustained imbalance between muscle protein synthesis and protein degradation and the absent response to nutrients eventually lead to muscle atrophy and loss of lean body mass (43), which is associated with increased morbidity during acute illness (10; 20) and with growth failure in children (7). Therefore, the molecular mechanisms that regulate protein synthesis and degradation in muscle during sepsis and inflammation, independent of nutrient and anabolic stimulation, are important therapeutic targets to limit the loss or promote recovery of skeletal muscle mass in these conditions.

In neonatal animals, whole body protein turnover is very high (38), likely to allow remodeling and rapid growth of skeletal muscle, as muscle mass is the most abundant and rapidly growing tissue in the body (3). Rapid growth in neonatal animals occurs not only because neonates are efficient in utilizing nutrients for protein deposition in muscle, but also in large part due to higher baseline muscle protein synthesis rates than mature animals. Anabolic
agents such as insulin and AA stimulate muscle protein synthesis, but also oppose muscle protein degradation in mature animals. In the immediate post-natal period, muscle protein synthesis declines rapidly with development (3), but it is not known whether development also influences muscle protein catabolism as the animal grows from the neonatal phase. The molecular regulation of muscle degradation has been reported from studies in tissue culture and mature rodents or humans, but less is known of the regulation of degradation in the immature individual. Moreover, it is not known whether development influences the activation of signaling pathways associated with protein degradation in skeletal muscle when sepsis induces protein catabolism.

Protein degradation in muscle presents a number of signaling components that are controlled by regulators that are also involved in the translational control of protein synthesis (9). The 5’-AMP-activated protein kinase (AMPK), a sensor of cellular energy, is activated by rising AMP levels as a result of energy starvation, and results in mTOR inhibition, thus restraining protein synthesis (31). In skeletal muscle, AMPK also increases the expression of the E3 ubiquitin ligases, muscle atrophy F-box (MAFbx, atrogin1) and muscle RING finger 1 (MuRF1), suggesting that AMPK may also be important for the regulation of skeletal muscle wasting (13; 37). Atrogin1 and MuRF1 have been associated with protein degradation by the ubiquitin-proteosomal pathway in muscle (5; 19; 22).

PKB (protein kinase B), also known as Akt, is a signaling protein involved in insulin signaling transduction and regulation of protein synthesis that has been shown to inhibit caspase-3 activity. Caspase-3 facilitates the destruction of intact muscle fibers and produces substrates (e.g., protein fragments, ATP) for the ubiquitin-proteasome system to increase the proteosomal degradation capacity (8). PKB also regulates protein degradation in muscle by phosphorylating and inactivating nuclear transcription factors termed Forkhead box 0 (Fox0) (22).
Phosphorylation and inactivation of Fox0s result in their export from the nucleus and translocation to the cytoplasmic compartment (19; 22), leading to inactivation of atrogin1 and MuRF1 transcription and a decrease in protein degradation. Cytokines such as TNF-α induce the activation of the Rel/NF-B (NF-B) family of transcription factors (42), which have been shown to induce loss of skeletal muscle proteins, as measured by increases in amino acid excretion and tyrosine turnover in isolated muscles (1), possibly by up regulation of MuRF1, but not atrogin1 (9). Other publications suggest that cytokines such as TNF-α induce atrogin1 in a p38MAPK dependent mechanism (17), indicating that cytokines can induce protein degradation by up regulating the expression of E3 ligases. To assess muscle degradation rates in vivo, most of the methods available are estimations of mobilization of nitrogen and muscle components, such as 3-methylhistine (39) and α-actin (18; 44), and they have too many technical and biological limitations to be considered a gold standard (34).

Since the anabolic drive in the neonatal animal declines with development, we hypothesize that skeletal muscle protein degradation induced by bacterial endotoxemia becomes more severe as the neonate develops. While we have previously examined the effects of endotoxin on the response of muscle protein synthesis to insulin and AA stimulation in the neonatal pig, in the current study, we wish to understand the intrinsic mechanistic alterations caused by endotoxin on the regulation of muscle protein degradation, without the antagonism of anabolic stimulatory conditions. We infused fasted neonatal (7-d-old) and 26-d-old pigs with Escherichia coli endotoxin (lipopolysaccharide, LPS) and measured muscle degradation markers and signaling proteins that control protein degradation in the longissimus dorsi (LD) muscle. The dose of LPS chosen was based on our previous studies on LPS porcine models which allow animal survival, and elicit a hyperdynamic septic-like response with hyperthermia and
hypercytokinemia. The results suggest that skeletal muscle in the healthy neonatal pig maintains high baseline degradation signal activation that cannot be enhanced by LPS, but as maturation advances, the effect of LPS on muscle protein catabolism increases in severity.

MATERIALS AND METHODS

Animals. One to two weeks before farrowing, four pregnant crossbred sows (Landrace x Yorkshire x Hampshire x Duroc) obtained from the Agriculture Headquarters of the Texas Department of Criminal Justice (Huntsville, TX), were housed in environmentally controlled lactation crates and provided with water ad libitum and a commercial diet (5084, PMI Feeds, Richmond, IN). After farrowing, the piglets were allowed to reside with the sow and were not given supplemental creep feed. Twenty-six neonatal piglets resided and suckled from the sows until studied at 6-7 days of age (n=12, 2.5 ± 0.2 kg body weight) and at 26 days of age (n=14, 7.8 ± 0.8 kg body weight). Three days prior to the experiment, the piglets were anesthetized for sterile catheter insertion into a jugular vein and a carotid artery, and returned to the sow until studied. The protocol, previously described in Orellana et al (29), was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Experimental design. Pigs were randomly assigned to control (7-d-old, n=5; 26-d-old, n=7) or LPS (7-d-old, n=7; 26-d-old, n=7) treatment groups. After 14-16 h of fasting, pigs were placed in a sling restraint system, and concentrations of whole blood glucose and plasma insulin were obtained. The LPS group received a continuous infusion (10 µg•kg⁻¹•h⁻¹) of LPS (lyophilized E. coli Serotype 0111-B4, Sigma Chemical Co., St. Louis, MO) for 8 h while the control group received an equal volume of sterile normal saline solution. Heparinized blood
samples were obtained at 1 h intervals to determine whole blood glucose concentrations by automated glucose oxidase reaction (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH). Plasma was aliquoted and stored at -70°C until insulin concentrations were measured by a porcine insulin RIA kit (Linco, St. Charles, MO) and individual plasma AA and 3-MH concentrations were measured with an HPLC method (PICO-TAG reverse-phase column; Waters, Milford, MA) as previously described. α-Actin in plasma was determined by protein electrophoresis and antibody immunoblotting as described below, and its abundance was normalized by the total protein content of the plasma sample, which was determined using bicinchoninic acid (36). All animals were euthanized with an intravenous dose of pentobarbital sodium (50 mg/kg body wt) 8 h after the LPS infusion began.

**Protein immunoblot analysis.** Muscle homogenates were separated on polyacrylamide gel electrophoresis (PAGE) and were analyzed at the same time in triple-wide gels (C.B.S. Scientific C., Del Mar, CA) for protein electrophoresis and antibody immunoblotting as previously described (26). Phosphorylated forms of the signaling proteins were compared to their abundance, i.e., the total content of the respective protein, for normalization. Non-phosphorylated forms of the signaling proteins in muscle were compared to the abundance of β-actin. The antibodies used in the immunoblotting process were PKB (total and Ser473, Cell Signaling Technology, Beverly, MA), AMPK-α (total and Thr172, Cell Signaling), Fox01 (total protein, Santa Cruz Technology and Ser256, Cell Signaling), Fox04 (total protein and Ser262, Cell Signaling), β-actin (total protein, Cell Signaling), NFκB (total and Ser536, Cell Signaling), caspase-3 (full length caspase-3 [pro-caspase, 35 kDa], and cleaved fraction [activated caspase-3, 17 kDa], Cell Signaling) (16), MuRF1 (total protein, ECM Biosciences, Versailles, KY),
atrogin1 (total protein, ECM Biosciences), and α-actin (full length 42 kDa protein and 14 kDa cleaved fraction, DakoCytomation, Denmark).

Statistical analyses. Analysis of variance (general linear modeling, GLM; Tuckey between group analyses) was used to assess the interaction between LPS and maturation on muscle protein degradation markers (Minitab Inc. for Windows). Treatment was the grouping factor for different parameters. If an effect was found by GLM, the difference among groups was determined by one-way ANOVA. Results are presented as means ± SE. Probability values of <0.05 were considered statistically significant.

RESULTS

Circulating hormone and substrate concentrations in response to LPS. Circulating insulin concentrations in response to 8 h of endotoxin in 7- and 26-d-old pigs were reported previously (29) and are presented here for reference. After a 14-16 h of fasting, baseline plasma insulin levels were higher in 26- than in 7-d-old pigs (Controls: 7-d-old: 1.8 ± 0.5, 26-d-old: 3.1 ± 0.7; LPS: 7-d-old: 1.5 ± 1.0, 26-d-old: 2.4 ± 0.4 µU/ml; P < 0.05). LPS induced a more robust insulin elevation in the 26-d-old animals compared to 7-d-old pigs (7-d-old: control: 1.6 ± 0.3, LPS: 1.0 ± 0.1; 26-d-old: control: 4.2 ± 1.1, LPS: 9.5 ± 1.9 µU/ml; P < 0.05). We have reported in previous studies using pancreatic-substrate clamps that fasting insulin concentrations in the normal newborn pig are below 5 µU/ml (27).

LPS raised circulating concentrations of total AA in 26-d-old pigs mainly by increasing non-essential AA (NEAA) (P < 0.05, Table 1). While LPS raised circulating concentrations of total NEAA in 7-d-olds as well, this effect was more robust in the 26-d-olds, thus interaction between age and the response to LPS was not found, except for ornithine and proline. Plasma
levels of some NEAA, such as cysteine, glutamine, glycine, and proline were higher and tyrosine concentrations were lower in 26- than 7-d-old pigs. LPS increased plasma concentrations of alanine, asparagine, citrulline, glutamine, ornithine, proline, tyrosine and taurine (P < 0.05, Table 1). Concentrations of many EAA, such as arginine, isoleucine, leucine, threonine, and valine, were lower in 26- than 7-d-old pigs. LPS induced a decrease in arginine, isoleucine, leucine, and lysine concentrations in 7-d-old pigs, increased circulating histidine in both age groups, and raised lysine concentrations in 26-d-old pigs only. BCAA (BCAA; isoleucine, leucine and valine) were lower in 26- compared to 7-d-old pigs, and LPS reduced BCAA in the 7-, but not in the 26-d-old pigs. Since LPS decreased many EAA in 7-, but increased some others in 26-d-old pigs, we found interaction between the modification imposed by age and the response to LPS on EAA, but not for EAA (P < 0.05, Table 1). Since the final blood sampling occurred after the tracer infusion and phenylalanine concentrations were greatly altered by the ³⁵H-phenylalanine used as a tracer, the sampling for phenylalanine occurred only before the tracer infusion.

3-MH and full length α-actin in plasma in response to LPS. Plasma 3-MH in 7-d-old pigs was lower in the presence of LPS. In contrast, LPS increased plasma 3-MH in 26-d-old pigs only, indicating an interaction between age and LPS (P < 0.05, Fig 1A). Plasma full length (42 kDa) α-actin abundance was greater in 7- than in 26 d-old pigs, but it was not affected by LPS. Similar to 3-MH, LPS increased the plasma full length α-actin abundance in 26-d-old pigs only (P < 0.05, Fig 1B).

PKB and AMPK phosphorylation in muscle in response to LPS in 7- and 26-d-old pigs. The relative phosphorylation of PKB (the phosphorylated form corrected by the total abundance of the protein) was higher in 26- when compared to 7-d-old pigs, likely as a consequence of the higher fasting insulin levels found in the older animals (P < 0.05, Fig. 2A). 26-d-old pigs had
lower PKB abundance when compared to 7-d-old pigs (P < 0.05, Fig. 2B). LPS did not affect PKB abundance or phosphorylation in either age group (Figs. 2A and B). LPS induced AMPK phosphorylation in both age groups (P < 0.05, Fig. 2C), which did not differ between 7- and 26-d-old pigs. AMPK abundance was not affected by LPS or age (P > 0.05, Fig. 2D).

*Fox0 phosphorylation in muscle in response to LPS in 7- and 26-d-old pigs.* Phosphorylation of Fox0s cause their inactivation in the nucleus, their release from DNA, and their translocation to the cytoplasmic compartment, decreasing the transcription of genes encoding proteins involved in protein degradation, such as atrogenes (22; 27). LPS decreased Fox01 and -4 phosphorylation in 26-d-old pigs (P < 0.05, Figs. 3A and B) suggesting activation of protein degradation. The abundances of both Fox0 proteins were not affected by age or LPS treatment (data not shown). 7-d-old pigs had higher Fox01 phosphorylation than 26-d-olds, which was not affected by LPS, in contrast to 26-d-olds (P < 0.05, Fig. 3A). Age did not affect Fox04 phosphorylation (P < 0.05, Fig. 3B).

*Activation of NFκB and caspase 3 in muscle in response to LPS in 7- and 26-d-old pigs.* LPS increased NFκB phosphorylation in both 7- and 26-d-old pigs (P < 0.05, Fig. 4A), but the response was more robust in the older pigs. The abundance of pro-caspase 3 was greater in 7- than in 26-d-old pigs. LPS decreased pro-caspase 3 abundance in 7-, but increased it in 26-d-old pigs, indicating an interaction between age and LPS (P < 0.05, Fig 4B). LPS increased the activated (cleaved) form of caspase 3 in both 7- and 26-d-old pigs. Activated caspase 3 was higher in 7- than 26-d-old control pigs (P < 0.05, Fig. 4C).

*Proteosomal degradation and cleavage of muscle α-actin in response to LPS in 7- and 26-d-old pigs.* LPS increased the abundances of E3 ubiquitin-ligases associated with proteosomal degradation, atrogin1 and MuRF1, in both age groups. Atrogin1 abundance was
higher in 26- compared to 7-d-old control pigs (P < 0.05, Fig. 5A and B). LPS increased the abundance of cleaved (14 kDa) α-actin in muscle in both age groups, and this response was more robust in 26 d-old pigs (P < 0.05, Fig 6A). Moreover, LPS increased plasma cleaved α-actin abundance in 26-d-old pigs only (P < 0.05, Fig 6B).

DISCUSSION

The sepsis-induced failures of the mechanisms that regulate muscle growth have been studied extensively in adult and mature organisms (15; 35). Muscle anabolic drive decreases as the animal matures (4), towards conditions of minimal synthesis and anabolic response (6). Given that the increase in muscle mass is greater than that of the body as a whole during the immediate post-natal growth (3), and that sepsis and inflammation accelerate degradation and decrease protein synthesis in mature muscle (10; 15; 32), it is reasonable to expect developmental modifications in muscle protein degradation induced by inflammation as individuals grow from the immediate post-natal period to maturity. Neonates possess a high anabolic drive in muscle to sustain rapid growth (3), which is driven by a post prandial protein synthesis rate that is higher than that of protein degradation and allows protein accretion in muscle (3; 25). In the neonate, high rates of muscle protein synthesis and high rates of protein turnover allow the AA released from the degradation of proteins to reincorporate into the muscle, a process recognized as muscle remodeling, which has been reported to occur in mature muscle but has not been investigated in relation to growth (9; 30). Using pigs as a model of the growing human neonate, we examined the intrinsic mechanistic alterations caused by endotoxin on the regulation of muscle protein degradation, without the antagonism of post-prandial anabolic
stimulation. Our study shows that the effect of LPS on muscle protein catabolism increases in severity as the neonate matures.

*LPS augments plasma total AA concentrations, 3-MH and full length α-actin in 26- but not in 7-d-old pigs.* In the current study, LPS induced higher fasting plasma insulin concentrations in 26-d-old pigs, concomitant to higher total AA concentrations, suggesting that insulin concentrations below the post-prandial range do not restrain the net total body AA efflux in the presence of LPS in more mature animals. In healthy neonatal pigs, insulin augments whole body AA disposal and induces a fall in total AA concentrations to allow protein deposition in muscle, but it does not affect liver protein synthesis (24; 27). In contrast, LPS increases insulin and decreases BCAA plasma concentrations and neonatal pigs (28; 40), likely due to LPS-induced pancreatic insulin secretion (14) and an increase in whole body AA utilization due to increased liver protein synthesis (26). Therefore, the appearance of higher total plasma AA concentrations in LPS-infused 26-d-old pigs may be driven by the systemic inflammatory response.

In the current study, 7-d-old control pigs had higher BCAA and EAA plasma appearance in response to fasting when compared to 26-d-old pigs, suggesting that neonatal animals primarily mobilize EAA during food deprivation. Endotoxemia in neonates appears to preferentially deplete plasma EAA when compared to older pigs. In 26-d-old pigs, LPS induced a more robust rise in plasma NEAA, as well as elevation of AA that are considered nitrogen shuttles between muscle and liver (38). This may suggest that neonatal animals may require further EAA supplement in the presence of acute sepsis, while in more mature animals, in which EAA do not appear to be limiting, it may become necessary to reverse a more apparent state of
insulin resistance that does not restrain protein catabolism and nitrogen flux from skeletal muscle.

Since a significant fraction of the plasma AA pool comes from tissues other than muscle, correlation among several muscle markers are needed to interpret a catabolic response. Similar to the increase in total AA in 26-d-old pigs in plasma, LPS increased the appearance of 3-MH and full length and cleaved α-actin. 3-MH, a component of the myofibrils that is liberated when the muscle structure is damaged or degraded, has been linked to proteolysis and muscle degradation in humans (33; 41), and released 3-MH is not metabolized and is excreted in the urine (34). In contrast to 26-d-olds, LPS reduced plasma 3-MH in 7-d-old pigs, similar to the drop seen in BCAA. Since 3-MH can be stored as balanine in pig muscle, but not in humans (33), such reductions may suggest that accretion of 3-MH and BCAA in muscle was not impaired by LPS in neonatal pigs. Similarly, LPS did not affect plasma full length (42 kDa) α-actin abundance in of 7-d-old pigs. Since full length α-actin abundance in plasma was higher in 7- than in 26-d-olds, the results may indicate that the higher degradation rates and rapid muscle turnover required to sustain protein accretion, muscle remodeling, and the rapid increase in muscle mass characteristic of the neonatal period (9) cannot be accelerated by LPS in neonatal pigs. α-actin is also a component of the muscle fiber that is liberated when the muscle structure is damaged or degraded, and has been linked with muscle damage during injury in animals (18; 44). Some had advocated that the cleaved fraction of α-actin (14 kDa) can be used as a tool to assess muscle protein degradation in humans (44). Although α-actin in plasma may derive from tissues other than muscle, in our study, LPS increased cleaved α-actin abundance in both muscle and plasma of 26-d-old pigs, supporting increased activation of degradation signaling in muscle. All together, these findings suggest a high muscle turnover rate in healthy neonatal pigs that is not
augmented by LPS, distinct from accelerated muscle degradation induced by LPS in more mature animals.

*LPS impairs cellular energy and activates proteosomal degradation signaling in muscle.*

We have shown for the first time that LPS augmented the relative phosphorylation of AMPK, an event associated with low energetic balance in the cell (23). AMPK phosphorylation or abundance was not affected by age, suggesting that the effects of LPS on AMPK signaling are specific to endotoxin (23), and not modified by development. Increased AMPK phosphorylation has also been associated with activation of proteolytic pathways in muscle (13; 23). AMPK activation during experimental sepsis has been shown to increase Fox01 mRNA transcripts in cell culture (23), which has been associated with activation of protein degradation by the ubiquitin-proteosomal pathway in muscle (21). Similar to AMPK, the LPS-associated increased in the abundance of E-3 ligases coupled with proteosomal degradation, MuRF1 and atrogin1, in both 7- and 26-d-old pigs, suggest events specific to sepsis and not modified by development.

In 26-d-old pigs, LPS reduced the phosphorylation of Fox01 and -4, which enhances proteolytic activity. While age did not affect Fox04 phosphorylation, 7-d-old pigs had higher Fox01 phosphorylation than 26-d-olds, despite higher insulin concentrations and increased PKB phosphorylation in muscle (22). It has been suggested that insulin regulates protein degradation via atrogin 1 expression through PKB and Fox01, while Fox04 responds to inflammatory mediators such as TNF-α (21). In our study, even though PKB phosphorylation was relatively higher in 26-d-old when compared to neonatal pigs, physiologically the animals remained in a fasted, non-stimulatory state, in which PKB is not able to stimulate translation signaling due to fasting insulin concentrations (29). Moreover, PKB phosphorylation did not change with LPS administration in 26-d-old pigs despite the LPS-induced elevation in insulin levels, suggesting
decreased susceptibility to inactivation of Fox0 proteins in response to insulin in more mature animals when compared to neonates. The results imply that, in conditions lacking nutrient stimulation, LPS can induce signal activation of protein degradation in skeletal muscle in neonatal and older animals, and some of these events may suggest a point for insulin resistance downstream of PKB in the more mature animals.

**LPS activates NFκB and increases activated caspase 3 abundance in muscle.** LPS increased NFκB phosphorylation and cleaved caspase 3 abundance in both age groups, suggesting an intrinsic degradative pathway triggered by inflammation and damage (1; 30) and this was not affected by development. In this regard, greater abundance of pro-caspase 3 in 7-d-old pigs may imply a larger myofibrillar degradation machinery in muscle of the 7- compared to the 26-d-old pig (16). The decrease in pro-caspase 3 in response to LPS in muscle of 7-d-old pigs suggests an ability of the neonatal pig muscle to maintain muscle mass in the presence of LPS, and this evidence is supported by a lack of change in plasma total AA and α-actin and decreased 3-MH.

**Perspectives and significance.** The results of the current study suggest that in conditions lacking anabolic stimulation, LPS induces intrinsic activation of proteolytic pathways in skeletal muscle of pigs, which may be associated with cellular energetic impairment, as it has been demonstrated in muscle during human sepsis (32). Furthermore, high baseline degradation signal activation in neonatal pig muscle cannot be enhanced by endotoxin, but as maturation advances, the effect of LPS on muscle protein catabolism manifests its severity (29). Moreover, the lack of reduction of muscle degradation signals in the presence of the LPS-induced elevation in circulating insulin in 26-d-old pigs suggests insulin resistance to ameliorate protein catabolism in skeletal muscle that becomes evident as the animal grows from the neonatal period.
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FIGURE LEGENDS

**Figure 1.** Plasma 3-methylhistidine (3-MH) concentration (A) and full length (42 kDa) α-actin abundance (B) in 7- and 26-d-old pigs infused with LPS for 8 hr. 3-MH was measured by HPLC and α-actin abundance was estimated by Western Blot and normalized to the total protein content in the aliquot. Values are means ± SE; n=5-7/treatment/age. a,b,c: values with different superscript differ significantly (P < 0.05).

**Figure 2.** PKB and AMPK phosphorylation (A, C respectively) and abundances (B, D respectively) in skeletal muscle of 7- and 26-d-old pigs infused with LPS for 8 hr. Amounts of the phosphorylated (p) forms of each protein were normalized to its abundance (t), which is the total protein content recovered from the immunoblot. Values are means ± SE; n=5-7/treatment/age. a,b: values with different superscript differ significantly (P < 0.05).

**Figure 3.** Fox01 (A) and Fox04 (B) phosphorylation in skeletal muscle of 7- and 26-d-old pigs infused with LPS for 8 hr. Amounts of the phosphorylated (p) forms of each protein were normalized to its abundance (t), which is the total protein content recovered from the immunoblot. Values are means ± SE; n=5-7/treatment/age. a,b: values with different superscript differ significantly (P < 0.05).

**Figure 4.** NFκB phosphorylation (A) and pro-caspase 3 (B) and activated (cleaved) caspase 3 abundances (C) in skeletal muscle of 7- and 26-d-old pigs infused with LPS for 8 hr. Amounts of the phosphorylated (p) forms of NFκB were normalized to its abundance (t), which is the total protein content recovered from the immunoblot. Pro-caspase 3 and cleaved caspase 3 abundances were normalized to total β-actin abundance recovered from the immunoblot. Values are means ± SE; n=5-7/treatment/age. a,b: values with different superscript differ significantly (P < 0.05).
Figure 5. Atrogin1 (A) and MuRF1 (B) abundances in skeletal muscle of 7- and 26-d-old pigs infused with LPS for 8 hr. Abundances were normalized to total β-actin abundance recovered from the immunoblot. Values are means ± SE; n=5-7/treatment/age. a,b,c: values with different superscript differ significantly (P < 0.05).

Figure 6. Cleaved (14 kDa) α-actin abundance in muscle (A) and plasma (B) in 7- and 26-d-old pigs infused with LPS for 8 hr. α-Actin abundance was estimated by Western blot and normalized to the total protein content in the aliquot. Values are means ± SE; n=5-7/treatment/age. a,b,c: values with different superscript differ significantly (P < 0.05).


31. **Pruznak AM, Kazi AA, Frost RA, Vary TC and Lang CH.** Activation of AMP-activated protein kinase by 5-aminimidazole-4-carboxamide-1-beta-D-ribonucleoside


Fig. 1

A  Plasma 3-methyl histidine

B  Plasma full length α-actin abundance

LPS  nmol/mL  Arbitrary Units
7 d-old  26 d-old  7 d-old  26 d-old
-  a  a  a
+  b  a  b
7 d-old  26 d-old  7 d-old  26 d-old
-  a  a  c
+  b  b  c
Fig. 2

(A) PKB phosphorylation

(B) PKB abundance

(C) AMPK phosphorylation

(D) AMPK abundance
Fig. 3

A  Fox01 phosphorylation

B  Fox04 phosphorylation

Arbitrary Units

Arbitrary Units

LPS

LPS

7 d-old  26 d-old

7 d-old  26 d-old
**Fig. 4**

**A**  
NFκB phosphorylation  
- LPS  
7 d-old  
26 d-old

**B**  
Pro-caspase 3 abundance  
- LPS  
7 d-old  
26 d-old

**C**  
Activated (cleaved) caspase 3  
- LPS  
7 d-old  
26 d-old
Fig. 5

A

Atrogin1 abundance

Arbitrary Units

LPS

7 d-old

26 d-old

b

b, c

A

B

MuRF1 abundance

Arbitrary Units

LPS

7 d-old

26 d-old

b

b
Fig. 6

A. Muscle cleaved α-actin

B. Plasma cleaved α-actin
Table 1. Amino acid concentrations in fasted 7-d-old and 26-d-old pigs after 8 hr of LPS infusion.

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>7 d-old Controls</th>
<th>LPS</th>
<th>26 d-old Controls</th>
<th>LPS</th>
<th>Treatment effect</th>
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<tbody>
<tr>
<td>Arginine</td>
<td>170 ± 5</td>
<td>107 ± 8*</td>
<td>95 ± 7‡</td>
<td>96 ± 12</td>
<td>‡ * #</td>
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<tr>
<td>Histidine</td>
<td>55 ± 6</td>
<td>86 ± 7*</td>
<td>64 ± 7</td>
<td>102 ± 19*</td>
<td>*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>242 ± 16</td>
<td>169 ± 10*</td>
<td>111 ± 12</td>
<td>137 ± 19</td>
<td>‡ #</td>
</tr>
<tr>
<td>Leucine</td>
<td>249 ± 10</td>
<td>195 ± 6*</td>
<td>171 ± 12‡</td>
<td>207 ± 22</td>
<td>‡ #</td>
</tr>
<tr>
<td>Lysine</td>
<td>225 ± 10</td>
<td>178 ± 12</td>
<td>158 ± 13</td>
<td>262 ± 22*‡</td>
<td>* #</td>
</tr>
<tr>
<td>Methionine</td>
<td>57 ± 8</td>
<td>50 ± 4</td>
<td>43 ± 6</td>
<td>65 ± 11</td>
<td>#</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>71 ± 11</td>
<td>73 ± 5</td>
<td>110 ± 6‡</td>
<td>162 ± 17*‡</td>
<td>‡ * #</td>
</tr>
<tr>
<td>Threonine</td>
<td>166 ± 20</td>
<td>123 ± 7</td>
<td>121 ± 2</td>
<td>84 ± 9</td>
<td>‡ *</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>37 ± 4</td>
<td>43 ± 5</td>
<td>52 ± 1‡</td>
<td>66 ± 10‡</td>
<td>‡</td>
</tr>
<tr>
<td>Valine</td>
<td>405 ± 14</td>
<td>331 ± 24*</td>
<td>239 ± 5</td>
<td>274 ± 28</td>
<td>‡ #</td>
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<table>
<thead>
<tr>
<th>NEAA</th>
<th>7 d-old Controls</th>
<th>LPS</th>
<th>26 d-old Controls</th>
<th>LPS</th>
<th>Treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>212 ± 35</td>
<td>323 ± 26</td>
<td>236 ± 51</td>
<td>544 ± 117</td>
<td>*</td>
</tr>
<tr>
<td>Asparagine</td>
<td>57 ± 5</td>
<td>80 ± 6</td>
<td>55 ± 5</td>
<td>83 ± 12</td>
<td>*</td>
</tr>
<tr>
<td>Citrulline</td>
<td>80 ± 3</td>
<td>93 ± 19</td>
<td>72 ± 13</td>
<td>111 ± 16</td>
<td>*</td>
</tr>
<tr>
<td>Cysteine</td>
<td>32 ± 2</td>
<td>34 ± 3</td>
<td>37 ± 5</td>
<td>46 ± 6</td>
<td>‡</td>
</tr>
<tr>
<td>Glutamine</td>
<td>232 ± 11</td>
<td>349 ± 21</td>
<td>328 ± 30</td>
<td>424 ± 82</td>
<td>‡ *</td>
</tr>
<tr>
<td>Glycine</td>
<td>461 ± 31</td>
<td>433 ± 30</td>
<td>626 ± 100</td>
<td>678 ± 58</td>
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</tr>
<tr>
<td>Ornithine</td>
<td>78 ± 8</td>
<td>87 ± 8</td>
<td>51 ± 4</td>
<td>109 ± 20*</td>
<td>* #</td>
</tr>
<tr>
<td>Proline</td>
<td>150 ± 15</td>
<td>161 ± 18</td>
<td>176 ± 11</td>
<td>298 ± 55*‡</td>
<td>‡ * #</td>
</tr>
<tr>
<td>Serine</td>
<td>131 ± 10</td>
<td>118 ± 9</td>
<td>133 ± 16</td>
<td>146 ± 12</td>
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<tr>
<td>Taurine</td>
<td>60 ± 13</td>
<td>105 ± 11</td>
<td>49 ± 7</td>
<td>168 ± 70</td>
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<tr>
<td>Tyrosine</td>
<td>178 ± 36</td>
<td>229 ± 13</td>
<td>104 ± 6</td>
<td>222 ± 27</td>
<td>‡ *</td>
</tr>
</tbody>
</table>

| BCAA | 895 ± 21       | 695 ± 22*  | 521 ± 20‡  | 618 ± 17‡| ‡ * #           |
| EAA  | 1676 ± 99      | 1354 ± 24* | 1164 ± 93‡ | 1456 ± 90*| ‡ #             |
| NEAA | 1670 ± 127     | 2012 ± 74  | 1867 ± 203 | 2828 ± 300| ‡ *             |

| TOTAL AA | 3346 ± 153   | 3366 ± 79  | 3031 ± 215 | 4284 ± 366‡| * #             |

Values are means ± SE (nmol/ml); 7-d-old control, n=5; 7-d-old LPS, n=7; 26-d-old control, n=7; 26-d-old, n=7/group. Animals were fasted for 14-16 hr and infused with either LPS or normal saline solution for 8 hr. ‡ Different between ages; * Different between control and LPS; # Interaction between age and LPS. Interaction among groups tested by ANOVA, General Linear Model; comparisons between two groups by one-way ANOVA, P < 0.05. BCAA: branched-chain amino acids; EAA: essential amino acids; NEAA: non essential amino acids; AA: amino acids.