

Aspartate inhibits LPS-induced MAFbx and MuRF1 expression in skeletal muscle in weaned pigs by regulating Akt, AMPK α and FOXO1

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Abstract

Infection and inflammation can result in the rapid loss of muscle mass and myofibrillar proteins (muscle atrophy). In addition, aspartate (Asp) is necessary for protein synthesis in mammalian cells. We hypothesized that Asp could attenuate LPS-induced muscle atrophy in a piglet model. Twenty-four weaning piglets were allotted to four treatments, including non-challenged control, LPS challenged control, LPS+0.5% Asp and LPS+1.0% Asp. On d 21, the piglets were injected with i.p. injection of LPS (100 ug/kg BM) or saline. At 4 h post-injection, blood, gastrocnemius and longissimus dorsi muscles samples were collected for biochemical and molecular analyses. Asp decreased the concentrations of cortisol and glucagon in plasma. In addition, Asp increased protein and RNA contents in muscles, and decreased mRNA expression of muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1). Moreover, Asp decreased phosphorylation of AMPK α but increased phosphorylation of Akt and Forkhead Box O (FOXO) 1 in the muscles. Our results indicate that Asp suppresses LPS-induced MAFbx and MuRF1 expression via activation of Akt signaling, and inhibition of AMPK α and FOXO1 signaling.

Keywords

Aspartate, lipopolysaccharide, MAFbx, MuRF1, muscle atrophy, piglets

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Introduction

Skeletal muscle, the most abundant tissue in the body, plays an important role in supporting an animal's physical and biochemical activities.¹ The rapid loss of skeletal muscle mass and myofibrillar proteins can arise as a secondary symptom related to sepsis and inflammation, and consequently results in muscle atrophy.^{1,2} The stimulation of proteolysis observed during atrophy takes place, at least in part, owing to an activation of the ubiquitin-proteasome pathway.³ In the ubiquitin-proteasome pathway, the muscle-specific ubiquitin ligases muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) are considered to be the key regulators of muscle protein degradation.⁴ In the case of sepsis-induced atrophy, MAFbx and MuRF1 are up-regulated several fold.^{3,5} They can be regarded as the early markers of the atrophy process, facilitating the differential diagnosis of muscle diseases.^{3,5}

Several lines of evidence indicate that the signaling pathways, mediated by the nuclear transcription factors termed Forkhead Box O (FOXO), regulate the expression of the muscle-specific ubiquitin ligases (i.e. MAFbx and MuRF1).^{3,5} In skeletal muscle, the activation of FOXO has been identified in conditions of muscle atrophy.⁶ Various protein kinases, including

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Akt and AMPK, have been reported to regulate FOXO activation.^{1,3,5,7,8} Akt, a serine-threonine protein kinase, suppresses protein degradation through inhibiting the FOXO proteins from entering the nucleus and preventing transcription of FOXO target genes, i.e., MAFbx and MuRF1.⁶ AMPK, which is composed of one catalytic (α) and two non-catalytic subunits (β and γ), also controls protein degradation.⁸ Interestingly, unlike Akt, AMPK mediates muscle loss and catabolism through increasing the activity of FOXO transcription factors.⁸

Aspartate (Asp), which can be synthesized *de novo*, is categorized as a non-essential amino acid in mammals.⁹ Importantly, Asp has been shown to exert various functions in the physiological and biological processes. Firstly, Asp is used in the biosynthesis of nucleotides and proteins.¹⁰ Beyond its role as an amino acid in proteins, Asp provides carbon backbone for *de novo* pyrimidine biosynthesis and is necessary for the conversion of IMP to AMP in *de novo* purine biosynthesis.¹⁰ In addition, Asp can be metabolized by skeletal muscle, and its combinations with other substances play an important role on muscle metabolism.^{11,12} For example, Lancha et al.¹¹ reported that Asp and asparagine supplementation increased glycogen concentration and modulated the glucose uptake in rat muscle. Lancha et al.¹² also reported that Asp, asparagine, and carnitine supplementation increased the capacity of muscle to utilize free fatty acids and spare glycogen. However, there is little research on how Asp modulates muscle atrophy.

Accordingly, we hypothesized that Asp supplementation would protect against muscle atrophy through controlling the expression of muscle ubiquitin ligases. In this study, *Escherichia coli* LPS was administrated to mimic endotoxaemia.¹³ In addition, a piglet model was used to establish the model for studying nutrition and physiology of human, specifically child and adolescent with muscles undergoing rapid growth.^{14,15} Our objective was to evaluate whether Asp could inhibit LPS-induced muscle atrophy, and to elaborate its molecular mechanism(s).

Materials and methods

Animals and experimental design

All procedures performed on the animals were approved by the Animal Care and Use Committee of Wuhan Polytechnic University. In all, 24 weaned castrated barrows (Duroc \times Large White \times Landrace, weaned at 21 ± 1 d of age), weighing 8.9 ± 0.2 kg, were acquired. The piglet was individually housed in 1.80×1.10 m² pen at a temperature of 25–27°C, in a humid and light-controlled room with free access to food and water. The basal diet (Supplementary Table 1) was prepared on the basis of the nutrient requirements of National Research Council.¹⁶

The 24 piglets were randomly assigned to the following four treatments (six replicate pens for per treatment): (1) non-challenged control (CONTR; piglets fed a control diet and injected with 0.9% NaCl solution); (2) LPS-challenged control [LPS; piglets fed the same control diet and injected with *E. coli* LPS (*E. coli* serotype 055: B5; Sigma Chemical Inc., St. Louis, MO, USA)]; (3) LPS + 0.5% Asp treatment (piglets fed a 0.5% Asp diet and injected with LPS); and (4) LPS + 1.0% Asp treatment (piglets fed a 1.0% Asp diet and injected with LPS). The Asp doses (purity > 99%; Amino Acid Bio-Chemical Co., Wuhan, China) were selected according to our previous studies.^{17,18} Our previous studies showed that, prior to LPS challenge, dietary 0.5% and 1.0% Asp supplementation had no effect on growth performance, total and differential leukocyte counts, and serum biochemical parameters of weaning piglets,¹⁷ suggesting that the Asp level in basal diet was enough to meet the requirements of weanling piglets' growth and physiological function in normal physiological condition. However, our previous studies also showed that, after LPS challenge, both 0.5% and 1.0% Asp attenuated the alteration of these variables induced by LPS,¹⁷ suggesting that exogenous Asp supply exerted beneficial effects under pathological conditions. Thus, in this experiment, we focused on investigating the effect of dietary 0.5% and 1.0% Asp supplementation on muscle variables in LPS-challenged pigs, and did not investigate the effect of Asp in pigs without LPS challenge. We added 0.67%, 0.34% and 0% alanine (purity > 99%; Amino Acid Bio-Chemical Co.) to the control, 0.5% Asp and 1.0% Asp diets, respectively, to get isonitrogenous diets. After 19 d of feeding with the control, 0.5% Asp and 1.0% Asp diets, the challenged groups were treated with i.p. injection of LPS at 100 μ g/kg BM, and the non-challenged group was treated with the same volume of 0.9% NaCl solution. The experiment was conducted for 19 d, according to our previous study.¹⁹ In addition, the LPS dose was chosen in accordance with our previous experiments, which demonstrated that this dose of LPS caused tissue damage in weaning piglets.^{20,21}

Blood and muscle sample collections

At 4 h after administration with saline or LPS, blood samples were collected and centrifuged to separate plasma. Plasma was kept at -80°C for further measurement of cortisol and glucagon concentrations. Following blood collection, the piglets were humanely euthanized by i.m. injection of pentobarbital (80 mg/kg BM; Zao Zhuang Ke Shun Chemical Co., Ltd., Shandong, China). The gastrocnemius and longissimus dorsi (LD) muscles were collected under the semitendinosus and biceps flexor cruris (left hindlimb) and from the tenth rib (right side of the carcass), respectively.

The muscle samples were frozen immediately in liquid nitrogen, and then stored at -80°C for further measurement. In several studies, the gastrocnemius and LD muscles were selected to study muscle atrophy.^{22,23} High levels of MAFbx were found in the gastrocnemius and LD muscles in piglets with muscle fiber atrophy.²³ Therefore, we chose these two muscles to investigate muscle atrophy. In addition, the time point of 4 h following LPS or saline injection was selected according to the previous experiments.^{21,24,25}

Plasma cortisol and glucagon concentrations

Plasma cortisol and glucagon concentrations were measured with ^{125}I RIA assay kits (Beijing North Institute of Biological Technology, Beijing, China). All experimental procedures were performed according to the manufacturer's instructions.

Muscle protein, DNA and RNA contents

The methods to analyze muscle protein, DNA and RNA contents were the same as previously described.²⁰

Real-time PCR

RNA isolation, quantification, cDNA synthesis and real-time PCR were performed as previously described.²¹ The primer pairs used are presented in Supplementary Table 2. The $2^{-\Delta\Delta\text{CT}}$ method²⁶ was used to determine changes in gene expression between treatment groups with the GAPDH CT as the correction factor. We analyzed the stability of several housekeeping genes using the method of Vandesompele et al.²⁷ Our results demonstrated that the expression of GAPDH is more stable than that of other housekeeping gene, and GAPDH did not exhibit any difference among four treatments or two muscles. Relative mRNA abundance of each target gene was normalized to the control group.

Western blot

Western blot analysis was performed according to the method of Liu et al.²¹ Briefly, the muscle samples were homogenized and centrifuged, and the supernatants were collected to measure protein concentrations using the BCA reagent (Sigma Chemical Inc.). Equal amounts of muscle proteins (65 μg) were loaded onto polyacrylamide gels, separated through SDS-PAGE, transferred to blotting membranes, and then incubated with the primary antibodies (Abs). Next, the membranes were incubated with the secondary Ab. The following primary Abs were used: total AMPK α (tAMPK α ; 1:1000; #2532), phosphorylated AMPK α (pAMPK α , Thr172; 1:1000; #2535), total Akt (tAkt, 1:1000; #9272), phosphorylated Akt (pAkt, serine473; 1:1000; #9271), total

FOXO 1 (tFOXO1; 1:1000; #9454) and phosphorylated FOXO 1 (pFOXO1, serine256; 1:1000; #9461) (all from Cell Signaling Technology Inc., Danvers, MA, USA). An Enhanced Chemiluminescence Western blotting kit (Amersham, Little Chalfont, UK), a Gene Genome bioimaging system (Alpha Innotech, Kasendorf, Germany), and a GeneTools software (Syngene, Cambridge, UK) were used to develop, visualize and analyze the bands, respectively. Phosphorylated forms of AMPK α , Akt and FOXO1 were normalized with the total protein content.

Eight samples from four treatments ($n=2$) were run in one gel. Thus, 24 samples from four treatments ($n=6$) were run in three gels at one time to minimize the variations of gel to gel. The analyses were conducted in duplicate.

Statistical analysis

Data were analyzed by the mixed procedure of SAS (SAS Institute Inc., Cary, NC, USA) appropriate for a randomized complete-block design. The comparison between CONTR and LPS (0% Asp) group was to determine the effect of LPS on the piglets. Linear and quadratic polynomial contrasts were used to determine the response to dietary Asp supplementation among LPS-challenged piglets. The data were expressed as means and pooled SEM in Tables 1–3, or as means and SEs in Figures 1–4. The statistical significance level for all analyses was set at $P \leq 0.05$, and $0.05 < P \leq 0.10$ were considered as trends.

Results

Plasma cortisol and glucagon concentrations

Relative to CONTR piglets, LPS challenge increased the concentrations of cortisol and glucagon in plasma ($P \leq 0.001$; Table 1). Among the LPS-challenged piglets, Asp supplementation decreased the concentrations of cortisol (linear, $P < 0.05$; quadratic, $P < 0.05$) and glucagon (linear, $P < 0.05$) in plasma.

Protein, DNA and RNA contents

Compared with CONTR piglets, LPS challenge decreased DNA content in gastrocnemius and LD muscles ($P < 0.05$; Table 2). Among the LPS-challenged piglets, Asp supplementation increased protein (linear, $P < 0.05$; quadratic, $P < 0.05$) and RNA (linear, $P < 0.05$; quadratic, $P < 0.001$) contents in gastrocnemius muscle, and protein content in LD muscle (linear, $P < 0.05$).

mRNA expression of MAFbx and MuRF1

Relative to CONTR piglets, LPS challenge increased mRNA expression of *MuRF1* in gastrocnemius and LD muscles ($P < 0.05$; Figure 1), and *MAFbx* in LD

Table 1. Effects of Asp supplementation on plasma cortisol and glucagon concentrations in weaning piglets at 4 h after the administration of *E. coli* LPS challenge.^a

Item	Treatment				SEM	P-Value ^b		
	CONTR	LPS	LPS + 0.5% Asp	LPS + 1.0% Asp		LPS vs. CONTR	Linear	Quadratic
Cortisol (ng/ml)	62	241	193	188	12	<0.001	0.011	0.019
Glucagon (pg/ml)	126	316	292	234	24	0.001	0.041	0.118

^aValues are means and pooled SEM, $n = 6$ (one pig/pen).

^bThe LPS pigs were compared with CONTR pigs to determine the effect of LPS. Linear and quadratic polynomial contrasts were used to determine the response to Asp supplementation among LPS-challenged pigs.

Table 2. Effects of Asp supplementation on muscle protein, DNA and RNA contents in weaning piglets at 4 h after the administration of *E. coli* LPS challenge.^a

	Treatment					P-Value ^b		
Item	CONTR	LPS	LPS + 0.5% Asp	LPS + 1.0% Asp	SEM	LPS vs. CONTR	Linear	Quadratic
Gastrocnemius muscle								
Protein (mg/g tissue)	51	52	53	65	4	0.926	0.013	0.024
DNA (μg/g tissue)	87	61	73	74	10	0.043	0.361	0.604
RNA (μg/g tissue)	141	143	226	193	10	0.850	0.040	< 0.001
LD muscle								
Protein (mg/g tissue)	87	85	91	101	5	0.649	0.046	0.142
DNA (μg/g tissue)	143	77	67	54	13	0.012	0.145	0.355
RNA (μg/g tissue)	201	220	211	260	17	0.429	0.126	0.132

^aValues are means and pooled SEM, $n = 6$ (one pig/pen).

^bThe LPS pigs were compared with CONTR pigs to determine the effect of LPS. Linear and quadratic polynomial contrasts were used to determine the response to Asp supplementation among LPS-challenged pigs.

muscle ($P < 0.001$). Among the LPS-challenged piglets, Asp supplementation decreased mRNA expression of *MAFbx* in gastrocnemius (linear, $P < 0.05$) and LD (linear, $P = 0.095$; quadratic, $P = 0.086$) muscles, and *MuRF1* in gastrocnemius (linear, $P = 0.055$; quadratic, $P = 0.056$) and LD (linear, $P = 0.001$; quadratic, $P < 0.01$) muscles.

mRNA expression of AMPK α , Akt1 and FOXO1

Relative to CONTR piglets, LPS challenge increased mRNA expression of *FOXO1* in gastrocnemius and LD muscles ($P < 0.01$; Table 3). Among the LPS-challenged piglets, Asp supplementation increased mRNA expression of *AMPK α 1* in gastrocnemius muscle (linear, $P = 0.099$), and *AMPK α 2* in gastrocnemius (linear, $P < 0.01$; quadratic, $P < 0.05$) and LD (linear, $P < 0.05$) muscles.

Protein phosphorylation and abundance of AMPK α , Akt and FOXO1

Relative to CONTR piglets, LPS challenge decreased the ratio of pAkt/tAkt in gastrocnemius and LD

muscles, and the ratio of pFOXO1/tFOXO1 in LD muscle ($P < 0.05$; Figures 2–4). Among the LPS-challenged piglets, Asp supplementation decreased the ratio of pAMPK α /tAMPK α in gastrocnemius muscle, increased the abundance of tAMPK α in LD muscle, and increased the ratio of pAkt/tAkt and the ratio of pFOXO1/tFOXO1 in gastrocnemius and LD muscles (linear, $P < 0.05$; quadratic, $P < 0.05$).

Discussion

LPS is a potent endotoxin and acute inflammation induced by i.p. LPS injection can cause serious skeletal muscle wasting.^{28,29} LPS challenge has been shown to decrease the diameter of myofibers and myotubes, and increase myosin heavy chain degradation and induce the loss of muscle strength and mass in mice.³⁰ Our previous research showed that dietary addition of 0.5% and 1.0% Asp alleviated LPS-induced intestinal injury in weaning piglets.¹⁸ In this study, we extended our previous observations into the function of Asp in muscle tissue. Our results showed that Asp attenuated LPS-induced increase of plasma TNF- α ,¹⁸ cortisol and glucagon concentrations, suggesting that Asp

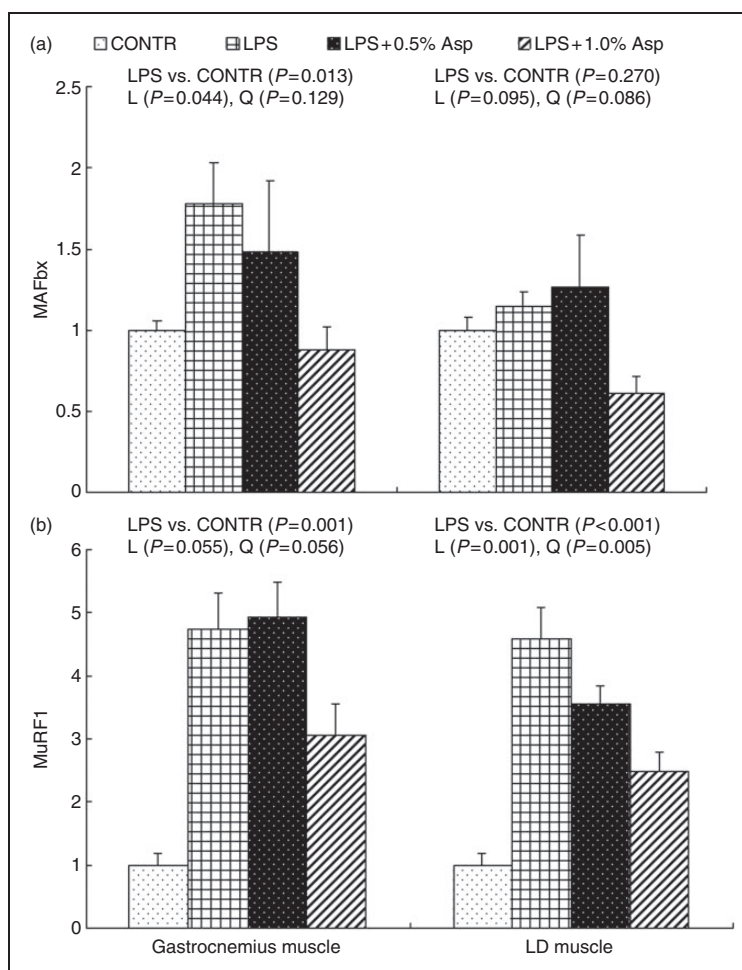


Figure 1. Effects of Asp supplementation on the mRNA expression of (a) MAFbx and (b) MuRF1 in gastrocnemius and LD muscles of weaning piglets at 4 h after LPS challenge. The LPS (0% Asp) pigs were compared with CONTR pigs (LPS vs. CONTR) to determine the effect of LPS. Linear (L) and quadratic (Q) polynomial contrasts were used to determine the response to dietary Asp supplementation among LPS-challenged pigs. Values are means with SEs, $n = 6$ (one pig/pen).

Table 3. Effects of Asp supplementation on muscle mRNA expression of AMPK α , AktI and FOXO1 in weaning piglets at 4 h after the administration of *E. coli* LPS challenge.^a

	Treatment					P-Value ^b		
Item	CONTR	LPS	LPS + 0.5% Asp	LPS + 1.0% Asp	SEM	LPS vs. CONTR	Linear	Quadratic
Gastrocnemius muscle								
AMPK α 1	1.00	0.96	1.17	1.30	0.19	0.887	0.099	0.261
AMPK α 2	1.00	0.97	1.33	1.58	0.14	0.886	0.003	0.014
Akt1	1.00	0.85	0.77	0.81	0.10	0.376	0.710	0.808
FOXO1	1.00	2.02	2.06	1.79	0.19	0.008	0.406	0.577
LD muscle								
AMPK α 1	1.00	1.05	1.21	1.39	0.15	0.790	0.134	0.338
AMPK α 2	1.00	0.95	1.09	1.37	0.13	0.682	0.042	0.126
Akt1	1.00	0.87	0.82	0.82	0.07	0.190	0.611	0.841
FOXO1	1.00	2.83	2.74	2.51	0.21	< 0.001	0.333	0.619

^{a,b}Values are means and pooled SEM, $n = 6$ (one pig/pen). The LPS pigs were compared with CONTR pigs to determine the effect of LPS. Linear and quadratic polynomial contrasts were used to determine the response to Asp supplementation among LPS-challenged pigs.

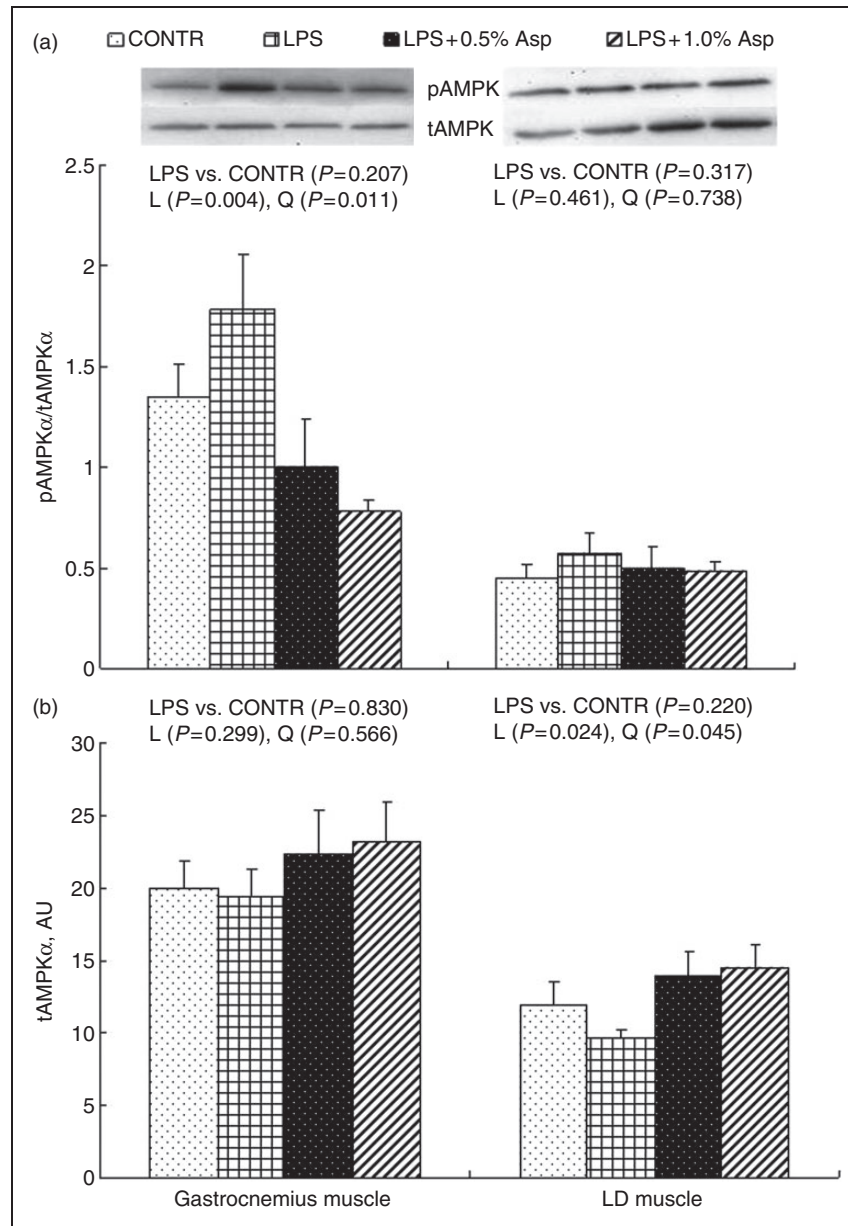


Figure 2. Effects of Asp supplementation on the (a) pAMPK α :tAMPK α ratio and (b) protein abundance of tAMPK α in gastrocnemius and LD muscles of weaning piglets at 4 h after LPS challenge. The bands shown are the representative Western blot images of pAMPK α (62 kDa) and tAMPK α (62 kDa). The LPS (0% Asp) pigs were compared with CONTR pigs (LPS vs. CONTR) to determine the effect of LPS. Linear (L) and quadratic (Q) polynomial contrasts were used to determine the response to dietary Asp supplementation among LPS-challenged pigs. Values are means with SEs, $n = 6$ (one pig/pen). AU: arbitrary units.

attenuates LPS-induced systemic inflammatory and stress responses.

Skeletal muscle atrophy during sepsis is primarily a result of accelerated protein degradation, and is characterized by the increased expression of the muscle-specific E3 ubiquitin ligases (MAFbx and MuRF1).³¹ MAFbx and MuRF1 are induced early in the atrophy process, and the increase in their mRNA expression precedes the loss of muscle mass.³² The muscle protein, RNA and DNA are common metrics for assessing the protein synthetic capacity, translational efficiency and

cell size, and they are negatively related to protein degradation.³³ In this study, we demonstrated that LPS injection increased mRNA expression of *MAFbx* and *MuRF1*, and decreased DNA content in muscles, indicating that LPS induced muscle atrophy. Our results are consistent with the findings of Schakman et al.,²⁸ in rats, and Jamart et al.,³⁴ in mice. Asp supplementation to the LPS-challenged piglets restored the protein and RNA contents, and decreased mRNA expression of *MAFbx* and *MuRF1* in muscles, suggesting that Asp attenuated LPS-induced muscle atrophy. To our best

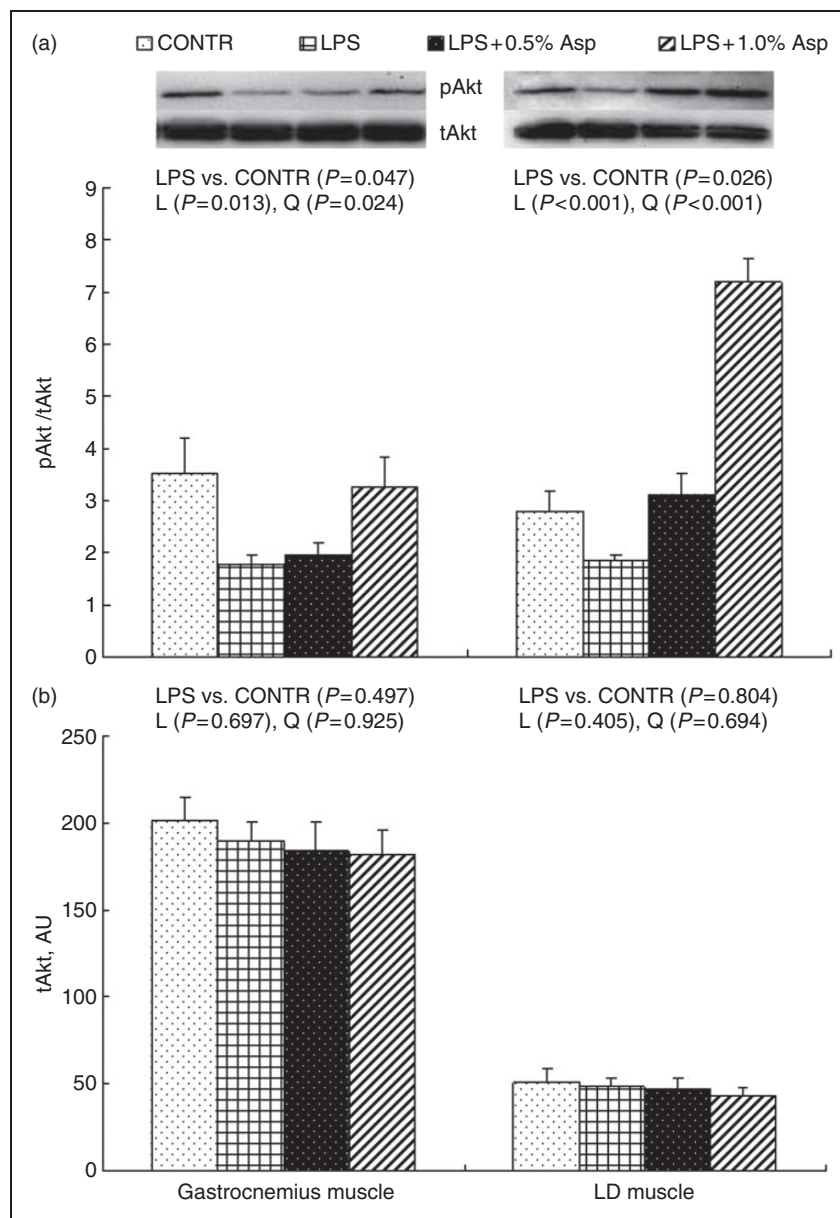


Figure 3. Effects of Asp supplementation on the (a) pAkt: tAkt ratio and (b) protein abundance of tAkt in gastrocnemius and LD muscles of weaning piglets at 4 h after LPS challenge. The bands shown are the representative Western blot images of pAkt (60 kDa) and tAkt (60 kDa). The LPS (0% Asp) pigs were compared with CONTR pigs (LPS vs. CONTR) to determine the effect of LPS. Linear (L) and quadratic (Q) polynomial contrasts were used to determine the response to dietary Asp supplementation among LPS-challenged pigs. Values are means with SEs, $n = 6$ (one pig/pen). AU: arbitrary units.

knowledge, our results demonstrated for the first time that Asp can alleviate muscle atrophy.

The family of FOXO transcription factor has been implicated in proteolysis during muscle atrophy.³⁵ In particular, FOXO1 participates in the regulation of MAFbx and MuRF1 expression in sepsis-induced muscle wasting.³⁶ Akt and AMPK are considered to regulate protein degradation in muscle through FOXO and FOXO target genes (i.e. MAFbx and MuRF1).^{1,3,5,7,8} Phosphorylation of Akt by insulin or insulin-like growth factor-1 reduces muscle proteolysis through phosphorylating and inactivating FOXO

transcription factors.³⁵ However, AMPK, in an active (phosphorylated) state, reduces the level of phosphorylated forms of FOXO and activates it.³⁷

In our study, LPS challenge decreased phosphorylation of Akt (the ratio of pAkt/tAkt) and phosphorylation of FOXO1 (the ratio of pFOXO1/tFOXO1). Similarly, Crossland et al.¹³ reported that during LPS-induced endotoxemia, Akt, in a predominantly dephosphorylated/inactive state, enabled FOXO factors to enter the nucleus and up-regulate MAFbx and MuRF1 expression, promoting ubiquitin-proteasome pathway-mediated protein degradation. Consistent

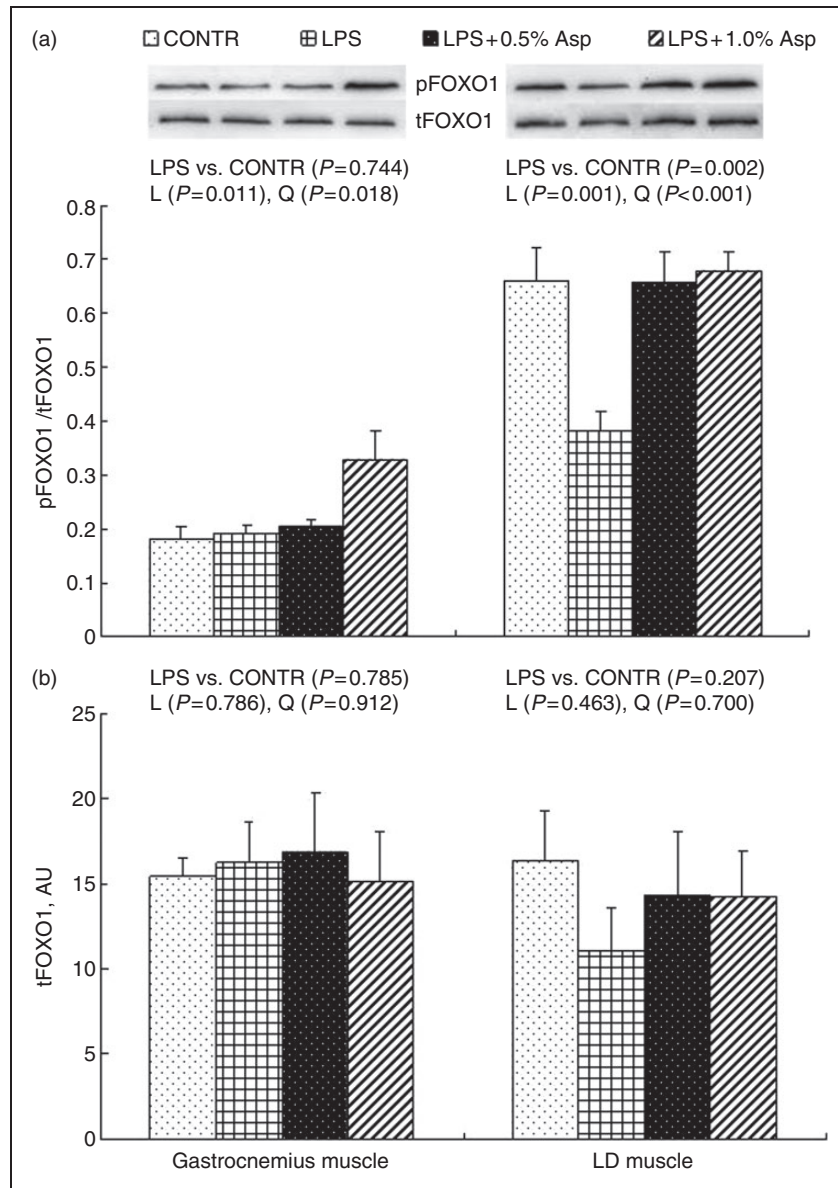


Figure 4. Effects of Asp supplementation on the (a) pFOXO:tFOXO ratio and (b) protein abundance of tFOXO in gastrocnemius and LD muscles of weaning piglets at 4 h after LPS challenge. The bands shown are the representative Western blot images of pFOXO (82 kDa) and tFOXO (82 kDa). The LPS (0% Asp) pigs were compared with CONTR pigs (LPS vs. CONTR) to determine the effect of LPS. Linear (L) and quadratic (Q) polynomial contrasts were used to determine the response to dietary Asp supplementation among LPS-challenged pigs. Values are means with SEs, $n = 6$ (one pig/pen). AU: arbitrary units.

with decreased mRNA expression of *MAFbx* and *MuRF1* in muscles, Asp supplementation to the LPS-challenged pigs increased phosphorylation of Akt and FOXO1, and decreased phosphorylation of AMPK α (the ratio of pAMPK α /tAMPK α). These results indicate that Asp prevented LPS-induced inhibition of Akt signaling and activation of AMPK α and FOXO signaling. So far, it is not known whether Asp directly or indirectly modulates muscle AMPK α , Akt and FOXO1 signaling. Asp may serve as substrate for protein synthesis or a signaling molecule, or both. Lancha et al.¹¹ reported that Asp combinations could

increase glycogen concentration in rat muscle. Glycogen can allosterically inhibit AMPK activity.³⁸ In this way, Asp might inhibit AMPK activity through increasing muscle glycogen concentration. In addition, it is also possible that Asp can be converted to other substances such as oxaloacetate³⁹ and arginine,⁴⁰ to regulate these signaling pathways. Recent studies have demonstrated that both oxaloacetate and arginine increased levels of phosphorylated Akt.^{41,42} Williams et al.⁴³ reported that oxaloacetate supplementation increased lifespan via an AMPK/FOXO-dependent pathway.

In our study, the results of Asp on some muscle measurements showed difference between gastrocnemius and LD muscles. This may be attributed to different anatomy and physiology among the various segments of muscles.⁴⁴ In addition, our observations found that mRNA and protein of some measurements were differentially expressed, which is consistent with the findings of Maier et al.⁴⁵ and Koussounadis et al.⁴⁶ The distinction may be related to different levels of regulation between transcript and protein product.^{45,46} For the proof of concept, we focused on samples collected only at one time point (4h) in LPS-induced muscle atrophy. In our future studies, we plan to conduct the measurements of multiple time points to further dissect the dynamic interplay and the long-term effects of Asp on muscle atrophy. Moreover, direct measurement of muscle mass and protein synthesis/degradation will be taken for the determination of the role of Asp on muscle growth.

In general, dietary Asp supplementation suppresses LPS-induced expression of muscle atrophy-associated genes and thus inhibits protein degradation. Asp may regulate muscle protein metabolism through controlling the expression of muscle ubiquitin ligases by regulating Akt, AMPK and FOXO1 signaling. However, according to our present study, it is difficult to draw the definite conclusion about how Asp affect the muscle atrophy. This is of concern and merits further investigation.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

- Fanzani A, Conraads VM, Penna F and Martinet W. Molecular and cellular mechanisms of skeletal muscle atrophy: an update. *J Cachexia Sarcopenia Muscle* 2012; 3: 163–179.
- Orellana RA, Suryawan A, Wilson FA, et al. Development aggravates the severity of skeletal muscle catabolism induced by endotoxemia in neonatal pigs. *Am J Physiol Regul Integr Comp Physiol* 2012; 302: R682–R690.
- Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 2005; 37: 1974–1984.
- Bodine SC, Latres E, Baumhueter S, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 2001; 294: 1704–1708.
- Mammucari C, Milan G, Romanello V, et al. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* 2007; 6: 458–471.
- White JP, Gao S, Puppa MJ, et al. Testosterone regulation of Akt/mTORC1/FoxO3a signaling in skeletal muscle. *Mol Cell Endocrinol* 2013; 365: 174–186.
- Nakashima K and Yakabe Y. AMPK activation stimulates myofibrillar protein degradation and expression of atrophy-related ubiquitin ligases by increasing FOXO transcription factors in C2C12 myotubes. *Biosci Biotechnol Biochem* 2007; 71: 1650–1656.
- Jaitovich A, Angulo M, Lecuona E, et al. High CO₂ levels cause skeletal muscle atrophy via AMP-activated kinase (AMPK), FoxO3a protein, and muscle-specific Ring finger protein 1 (MuRF1). *J Biol Chem* 2015; 290: 9183–9194.
- Corpeleijn WE, Riedijk MA, Zhou Y, et al. Almost all enteral aspartate is taken up in first-pass metabolism in enterally fed preterm infants. *Clin Nutr* 2010; 29: 341–346.
- Sullivan LB, Gui DY, Hosios AM, et al. Supporting aspartate biosynthesis is an essential function of respiration in proliferating cells. *Cell* 2015; 162: 552–563.
- Lancha AH Jr, Poortmans JR and Pereira LO. The effect of 5 days of aspartate and asparagine supplementation on glucose transport activity in rat muscle. *Cell Biochem Funct* 2009; 27: 552–557.
- Lancha AH Jr, Recco MB, Abdalla DS and Curi R. Effect of aspartate, asparagine, and carnitine supplementation in the diet on metabolism of skeletal muscle during a moderate exercise. *Physiol Behav* 1995; 57: 367–371.
- Crossland H, Constantin-Teodosiu D, Gardiner SM, et al. A potential role for Akt/FOXO signalling in both protein loss and the impairment of muscle carbohydrate oxidation during sepsis in rodent skeletal muscle. *J Physiol* 2008; 586: 5589–5600.
- Dunshea FR and Cox ML. Effect of dietary protein on body composition and insulin resistance using a pig model of the child and adolescent. *Nutr Diet* 2008; 65: S60–S65.
- Merrifield CA, Lewis M, Claus SP, et al. A metabolic system-wide characterisation of the pig: a model for human physiology. *Mol Biosyst* 2011; 7: 2577–2588.
- NRC. *Nutrient requirements of swine*, 10th ed. Washington, DC: National Academic Press, 1998.
- Shi HF, Liu YL, Li S, et al. Effect of Aspartic acid on growth performance, blood cell differential count, and blood biochemical measurements of weaned piglets after lipopolysaccharide challenge. *China J Anim Sci* 2013; 7: 38–43.
- Wang H, Liu Y, Shi H, et al. Aspartate attenuates intestinal injury and inhibits TLR4 and NODs/NF- κ B and p38 signaling in weaned pigs after LPS challenge. *Eur J Nutr* 2016; Feb 23 [Epub ahead of print].
- Pi D, Liu Y, Shi H, et al. Dietary supplementation of aspartate enhances intestinal integrity and energy status in weanling piglets after lipopolysaccharide challenge. *J Nutr Biochem* 2014; 25: 456–462.
- Liu Y, Huang J, Hou Y, et al. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. *Br J Nutr* 2008; 100: 552–560.
- Liu Y, Chen F, Odle J, et al. Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 signaling pathways in weaned pigs after LPS challenge. *J Nutr* 2012; 142: 2017–2024.
- Drew B, Phaneuf S, Dirks A, et al. Effects of aging and caloric restriction on mitochondrial energy production in gastrocnemius muscle and heart. *Am J Physiol Regul Integr Comp Physiol* 2003; 284: R474–R480.
- Ooi PT, da Costa N, Edgar J and Chang KC. Porcine congenital splayleg is characterised by muscle fibre atrophy associated with relative rise in MAFbx and fall in P311 expression. *BMC Vet Res* 2006; 2: 23.
- Meador BM, Krzyszton CP, Johnson RW and Huey KA. Effects of IL-10 and age on IL-6, IL-1 β , and TNF- α responses in

- mouse skeletal and cardiac muscle to an acute inflammatory insult. *J Appl Physiol* (1985) 2008; 104: 991–997.
25. Andreasen AS, Kelly M, Berg RM, et al. Type 2 diabetes is associated with altered NF- κ B DNA binding activity, JNK phosphorylation, and AMPK phosphorylation in skeletal muscle after LPS. *PLOS ONE* 2011; 6: e23999.
 26. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and $2^{-\Delta\Delta CT}$ method. *Methods* 2001; 25: 402–408.
 27. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3: RESEARCH0034.
 28. Schakman O, Dehoux M, Bouchuari S, et al. Role of IGF-I and the TNF α /NF- κ B pathway in the induction of muscle atrogenes by acute inflammation. *Am J Physiol Endocrinol Metab* 2012; 303: E729–E739.
 29. Zhang B, Yu C, Lin M, et al. Regulation of skeletal muscle protein synthetic and degradative signaling by alanyl-glutamine in piglets challenged with *Escherichia coli* lipopolysaccharide. *Nutrition* 2015; 31: 749–756.
 30. Morales MG, Olguín H, Di Capua G, et al. Endotoxin-induced skeletal muscle wasting is prevented by angiotensin-(1-7) through a p38 MAPK-dependent mechanism. *Clin Sci (Lond)* 2015; 129: 461–476.
 31. Morales MG, Abrigo J, Meneses C, et al. Expression of the Mas receptor is upregulated in skeletal muscle wasting. *Histochem Cell Biol* 2015; 143: 131–141.
 32. Shiota C, Abe T, Kawai N, et al. Flavones inhibit LPS-induced atrogen-1/MAFbx expression in mouse C2C12 skeletal myotubes. *J Nutr Sci Vitaminol (Tokyo)* 2015; 61: 188–194.
 33. Smith GI, Atherton P, Reeds DN, et al. Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women. *Clin Sci (Lond)* 2011; 121: 267–278.
 34. Jamart C, Gomes AV, Dewey S, et al. Regulation of ubiquitin-proteasome and autophagy pathways after acute LPS and epoxomicin administration in mice. *BMC Musculoskelet Disord* 2014; 15: 166.
 35. Cho JE, Fournier M, Da X and Lewis MI. Time course expression of Foxo transcription factors in skeletal muscle following corticosteroid administration. *Appl Physiol* (1985) 2012; 108: 137–145.
 36. Castellero E, Alamdari N, Aversa Z, et al. PPAR β/δ regulates glucocorticoid- and sepsis-induced FOXO1 activation and muscle wasting. *PLOS ONE* 2013; 8: e59726.
 37. Lee K, Ochi E, Song H and Nakazato K. Activation of AMP-activated protein kinase induce expression of FoxO1, FoxO3a, and myostatin after exercise-induced muscle damage. *Biochem Biophys Res Commun* 2015; 466: 289–294.
 38. Ha J, Guan KL and Kim J. AMPK and autophagy in glucose/glycogen metabolism. *Mol Aspects Med* 2015; 46: 46–62.
 39. Trudeau F. Aspartate as an ergogenic supplement. *Sports Med* 2008; 38: 9–16.
 40. Wu G, Bazer FW, Davis TA, et al. Important roles for the arginine family of amino acids in swine nutrition and production. *Livest Sci* 2007; 112: 8–22.
 41. Wilkins HM, Harris JL, Carl SM, et al. Oxaloacetate activates brain mitochondrial biogenesis, enhances the insulin pathway, reduces inflammation and stimulates neurogenesis. *Hum Mol Genet* 2014; 23: 6528–6541.
 42. Fujiwara T, Kanazawa S, Ichibori R, et al. L-arginine stimulates fibroblast proliferation through the GPRC6A-ERK1/2 and PI3K/Akt pathway. *PLOS ONE* 2014; 9: e92168.
 43. Williams DS, Cash A, Hamadani L and Diemer T. Oxaloacetate supplementation increases lifespan in *Caenorhabditis elegans* through an AMPK/FOXO-dependent pathway. *Aging Cell* 2009; 8: 765–768.
 44. Pessa JE, Zadoo VP, Garza PA, et al. Double or bifid zygomatic major muscle: anatomy, incidence, and clinical correlation. *Clin Anat* 1998; 11: 310–313.
 45. Maier T, Güell M and Serrano L. Correlation of mRNA and protein in complex biological samples. *FEBS Lett* 2009; 583: 3966–3973.
 46. Koussounadis A, Langdon SP, Um IH, et al. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci Rep* 2015; 5: 10775.