Effects of dietary leucine supplementation and immune system stimulation on plasma AA concentrations and tissue protein synthesis in starter pigs¹

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ABSTRACT: Immune system stimulation (ISS) adversely affects protein and AA metabolism and reduces productivity in pigs. Leucine (**Leu**) has a regulatory role in skeletal muscle protein turnover, which may be affected by ISS. The objective of this study was to evaluate the effects of ISS and dietary Leu supplementation on the protein fractional synthesis rate (**FSR**) of various tissues in pigs. Yorkshire barrows were surgically fitted with jugular vein catheters and assigned to one of three dietary treatments: (i) CON, 1.36% standardized ileal digestible (**SID**) Leu; (ii) LEU-M, 2.04% SID Leu; and (iii) LEU-H, 2.72% SID Leu. The diets were formulated to contain all essential AA 10% above estimated requirements for maximum whole-body protein deposition for this BW range. At the start of the 36-h challenge period (initial BW = 14.5 ± 0.8 kg), ISS was induced in pigs with lipopolysaccharide (**ISS+**; *n* = 7, 8, and 7 for CON, LEU-M, and LEU-H pigs, respectively); a subset of CON pigs was injected with sterile saline (**ISS-**; *n* = 6). During challenge period, pigs were fed every 4 h and feed intake of ISS- pigs was

kept equal to ISS+ pigs. At the end of the challenge period, FSR of liver, plasma, gastrocnemius, and LD proteins were determined with a flooding dose of L-[ring-²H₅]phenylalanine (40 mol%). All essential AA, most nonessential AA, and plasma urea-N peaked at 12 h and declined to baseline levels at 36 h after ISS was induced in ISS+ pigs (*P* < 0.05), whereas plasma AA and urea-N concentrations were constant in ISS- pigs. At 36 h, dietary Leu supplementation resulted in a linear decline in plasma isoleucine, valine, glutamine, and urea nitrogen concentrations (*P* < 0.05), whereas plasma Leu concentration was unaffected. Liver protein FSR was increased in ISS+ pigs (*P* < 0.05), whereas plasma and skeletal muscle protein FSR was not affected by ISS. Dietary Leu supplementation tended to diminish liver protein FSR (linear reduction; *P* = 0.052) and increase gastrocnemius protein FSR (linear increase; *P* = 0.085) in ISS+ pigs. Leucine supplementation above estimated requirements may support repartitioning of AA from visceral to peripheral protein deposition during ISS.

Key words: immune system stimulation, leucine, pigs, protein synthesis

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INTRODUCTION

The negative impact of infection and inflammation on animal growth and productivity is driven by distinct modifications in whole-body protein and AA metabolism (Reeds and Jahoor, 2001). These changes are mediated by pro-inflammatory cytokines that function in part to regulate protein synthesis and degradation in a tissue-specific manner. In general, immune system stimulation (**ISS**) leads to a decrease in skeletal muscle

protein synthesis and increases in muscle protein degradation and visceral protein synthesis, which together contribute to an overall lower rate of whole-body protein deposition (**PD**) and BW gain in growing animals (Johnson, 1997). During ISS, AA release from skeletal muscle and AA uptake by the liver are both elevated in order to support the inflammatory response, in particular hepatic gluconeogenesis and acute phase protein (**APP**) synthesis (Obled, 2003). While such short-term changes in AA partitioning are beneficial for mounting an immune response, prolonged alterations are undesirable as it negatively affects whole-body N utilization for PD.

The principal function of AA is to serve as substrates for protein synthesis. Among the essential AA (**EAA**), leucine (**Leu**) is a potent regulator of protein synthesis in skeletal muscle and other tissues through its effect on the mechanistic target of rapamycin complex 1 (**mTORC1**) signaling pathway (Saxton and Sabatini, 2017). As a consequence of its ability to integrate environmental, hormonal, and nutritional signals, mTORC1 is as much subject to positive regulation by Leu as it is to both direct and indirect negative regulation by pro-inflammatory cytokines (Frost and Lang, 2011). In general, mTORC1 activity is decreased in skeletal muscle and increased in the liver during ISS, which appears to mirror the changes in protein synthesis in these tissues. ISS may produce a specific and short-term Leu resistance in skeletal muscle that impairs the ability of this AA to stimulate translation initiation and protein synthesis through mTORC1 (Lang and Frost, 2004, 2005).

We previously determined dietary Leu supplementation reduced whole-body protein turnover in healthy pigs, but had no effect during ISS (Rudar et al., 2017). However, the contribution of visceral and peripheral tissues to whole-body protein turnover should be considered. Although Leu requirements for PD and the synthesis of APP during ISS do not increase per se, dietary Leu supplementation may still be beneficial in part due to its regulatory effects on skeletal muscle protein turnover. The objective of the current study was to evaluate the effects of ISS and dietary Leu supplementation on the fractional synthesis rate (**FSR**) of plasma, liver, and skeletal muscle protein with a flooding dose of phenylalanine (**Phe**) in starter pigs. We hypothesized that the reduction in skeletal muscle protein synthesis during ISS can be attenuated by dietary Leu supplementation.

MATERIALS AND METHODS

All procedures in the current study were conducted according to the Canadian Council on Animal Care (CCAC, 2009) and were approved by the University of Guelph Animal Care Committee.

Animals, Diets, and Treatments

The pigs used in the current study were the same animals described in Rudar et al. (2016). Briefly, jugular vein catheters for isotope infusion and serial blood collection were surgically placed in Yorkshire barrows (de Lange et al., 1989). Pigs were housed individually in metabolism crates and allowed to recover from surgery for 7 d. Pigs were randomly assigned to one of three dietary treatments: (i) **CON**, 1.36% standardized ileal digestible (**SID**) Leu (**SID** Leu: Lys = 100 [% Lys]); (ii) **LEU-M**, 2.04% **SID** Leu (**SID** Leu: Lys = 150); or (iii) **LEU-H**, 2.72% **SID** Leu (**SID** Leu: Lys = 200). The diets were formulated as isonitrogenous with the addition of L-Asp and L-Glu to the **CON** and **LEU-M** diets, and to contain all **EAA** 10% above estimated requirements for maximum PD for this BW range (Table 1). Pigs were fed every 4 h and at 2.2 times maintenance requirements for metabolizable energy (NRC, 2012), as described previously (Rudar et al., 2016). Daily feed allowance was low in order to avoid excessive feed refusals during ISS.

At the start of the 36-h challenge period (initial BW = 14.5 ± 0.8 kg), ISS was induced in pigs with a single intramuscular injection of *Escherichia coli* lipopolysaccharide (**LPS**; 30 µg/kg BW; strain 055:B5; Sigma-Aldrich Canada Co., Oakville, ON; ISS+; $n = 7, 8$, and 7 for **CON**, **LEU-M**, and **LEU-H** pigs, respectively). A subset of **CON** pigs was injected with sterile saline (ISS-; $n = 6$). During the challenge period, feed intake of **CON** pigs injected with saline was kept equal to pigs injected with **LPS**, as described previously (Rudar et al., 2016). Blood was collected just before feeding every 12 h at 0, 12, 24, and 36 h after ISS was induced for determining plasma AA and urea-N concentrations. Plasma was separated from whole blood after centrifugation ($1,500 \times g$ at 4 °C for 15 min) and stored at -20 °C until further analysis. At the end of the challenge period, pigs were euthanized by a lethal intravenous injection of sodium pentobarbital (0.3 mL/kg BW; Schering-Plough Canada, Inc., Kirkland, QC).

Tissue Protein Synthesis

At the end of the challenge period, and 36 h after the LPS injection, fractional rate of tissue protein synthesis was measured with a flooding dose of L-Phe (1.50 mmol/kg BW) containing L-[ring- $^2\text{H}_5$] Phe at 40 mol% (0.60 mmol/kg BW; Cambridge Isotope Laboratories, Inc., Tewksbury, MA). Pigs were infused with Phe 1 h after feeding, and blood samples (6 mL) were collected into EDTA blood collection tubes (BD Canada, Mississauga, ON) immediately before the start of the infusion and 5, 10, 20, and 30 min thereafter. A final blood sample (20 mL) was taken at 35 min after the start of the infusion and pigs were immediately euthanized with a lethal intravenous injection of sodium pentobarbital (0.3 mL/kg BW; Schering-Plough Canada, Inc.). Tissue samples of the liver, gastrocnemius muscle, and LD muscle were quickly removed, thoroughly rinsed with ice-cold saline, blotted dry, and snap frozen in liquid nitrogen. The exact duration (min) of Phe incorporation into tissue protein was recorded, from the start of the infusion to the time that samples were snap frozen. Tissue samples were stored at -80°C until further analysis.

Sample Preparation and Analysis

Diet AA content analysis was performed at the laboratory of Degussa AG (Hanau, Germany) by ion-exchange chromatography with postcolumn derivatization with ninhydrin (method 982.30; AOAC, 2006). Before protein hydrolysis, AA were oxidized with performic acid and neutralized with sodium metabisulphite (Fontaine, 2003). The samples were subsequently hydrolyzed with 6 M HCl for 24 h at 110°C , and AA were quantified with the internal standard method by measuring absorbance at 570 nm.

Plasma-free AA concentrations were analyzed using Ultra Performance Liquid Chromatography and with Empower Chromatography Data Software (Waters Corporation, Milford, CT) according to Boogers et al. (2008).

Plasma urea nitrogen (PUN) was analyzed using a commercial kit (Urea Nitrogen (BUN) Liqui-UV Test (Rate), Stanbio Laboratory, Boerne, TX). Briefly, 10 μL of urea standard (30 mg/dL) or sample was added in duplicate into wells of a 96-well plate. After the addition of the reagent, absorbance was measured immediately at 340 nm and 1 min thereafter at 37°C (Power Wave XS KC4, BIO-TEK Instruments, Inc., Winooski, VT). Plasma urea-N was calculated as the ratio between

the change in absorbance of sample and standard multiplied by the concentration of the standard. The interassay CV was 1% and the minimum detection level was 2.0 mg/dL.

Plasma and tissue samples were prepared for isotopic enrichment analysis according to Litvak et al. (2013). The isotopic enrichment of L-[ring- $^2\text{H}_5$] Phe in plasma- and tissue-free and protein-bound pools was determined by gas chromatography-mass spectrometry (GC-MS) as the *tert*-butyldimethylsilyl derivative (Burd et al., 2012). Volatile compound analysis was performed with a Scion 436 gas chromatograph linked to a Bruker triple quadrupole mass spectrometer operating in electron impact ionization mode (Bruker Ltd., Milton, ON). Calibration curves were prepared from purified L-[ring- $^2\text{H}_5$]Phe (Cambridge Isotope Laboratories, Inc.) for a low level of molar enrichment (0 to 1.5 mol%; protein-bound AA) and a high level of molar enrichment (0 to 30 mol%; plasma- and tissue-free AA). Ions with mass-to-charge ratios of 336 and 341 (corresponding to L-Phe and L-[ring- $^2\text{H}_5$]Phe, respectively) were monitored and converted to percentage molar enrichment. The interassay CV was 4.7% and 4.0% for the low and high molar enrichment calibration curves, respectively.

Calculations and statistical analysis. Protein FSR was calculated according to Garlick et al. (1980):

$$\text{FSR} = \frac{E_{\text{bound}} \times 1,440 \text{ min} / \text{d} \times 100\%}{E_{\text{free}} \times t}$$

where FSR (%/d) is defined as the percentage of tissue protein synthesized in one day, E_{bound} is the isotopic enrichment (mol%) of L-[ring- $^2\text{H}_5$]Phe in the protein-bound AA pool at time t , E_{free} is the isotopic enrichment (mol%) of L-[ring- $^2\text{H}_5$]Phe in the tissue-free AA pool at time t , and t (min) is the incorporation time of L-Phe into the protein-bound AA pool from the start of the infusion. Plasma protein FSR was calculated assuming that the precursor pool enrichment was equivalent to that in the liver-free AA pool.

Statistical analysis was conducted using the mixed model procedures of SAS (version 9.4; SAS Institute Inc., Cary, NC). Plasma AA and urea-N concentrations were analyzed as repeated measures across time with treatment, sampling time, pig, and block as sources of variation. Contrasts were constructed to assess the difference between ISS- and ISS+ pigs fed CON at 36 h and to evaluate the linear relationship between the SID Leu: Lys ratio and plasma AA and urea-N concentration in ISS+

pigs at 36 h postchallenge. Isotopic enrichment and FSR results were analyzed for treatment effects with block and pig as sources of variation; block and pig were considered random effects. When a significant treatment effect was detected, differences among individual means were assessed using the Tukey–Kramer post hoc test. Contrasts were constructed to assess the difference between ISS– and ISS+ pigs fed CON and to evaluate the linear relationship between the SID Leu: Lys ratio and tissue protein FSR in ISS+ pigs. In all analyses, individual pigs were the experimental unit. Results are expressed as least square means $\pm$ SE (largest value from repeated measures analysis selected as SE). Significance was accepted at $P < 0.05$ and probabilities between 0.05 and 0.10 were accepted as trends.

RESULTS

Prior to surgery, all pigs appeared healthy and readily consumed their daily feed allowance. The pigs recovered from surgery without complication and returned to presurgery feeding levels within 24 h after surgery. The ISS+ pigs displayed clinical signs of disease such as lethargy, diarrhea, and vomiting immediately after the LPS injection but recovered to at least 75% of their pre-challenge period feeding levels by 15 h after the LPS injection; this feeding level was maintained for the remainder of the challenge period. However, four pigs were removed prior to administration of the flooding dose and therefore were not included in the analysis (Rudar et al., 2016).

During the challenge period, plasma EAA, nonessential AA (NEAA), and branched-chain AA (BCAA; Figure 1) and urea-N (Figure 2) concentrations in ISS+ pigs peaked 12 h following ISS and declined to baseline levels 36 h thereafter, whereas there were no changes in plasma AA or urea-N concentrations over time in ISS– pigs. Plasma Leu concentration was not affected by dietary Leu supplementation, whereas there were linear decreases in plasma Ile, Val, Gln, and PUN concentrations with increasing dietary Leu supplementation in ISS+ pigs at 36 h (Supplemental Table 1 for EAA; Supplemental Table 2 for NEAA; $P < 0.05$).

Plasma and tissue protein FSR are presented in Table 2. There was no treatment effect on plasma-free Phe enrichment. Maximum plasma-free Phe enrichment was attained 5 min after the start of the infusion and was sustained thereafter (data not shown). The enrichment of free Phe in the liver, LD, and gastrocnemius ranged from 92% to 102% of that in plasma, respectively, indicating that

flooding conditions were successfully achieved and a constant rate of tracer incorporation into tissue protein over the 35-min labeling period (Davis and Reeds, 2001). There was no effect of either ISS or dietary Leu supplementation on plasma protein FSR. ISS increased liver protein FSR ($P < 0.05$), whereas liver protein FSR tended to decrease with increasing dietary Leu supplementation in ISS+ pigs ($P = 0.052$). There was no effect of ISS on gastrocnemius and LD protein FSR, but gastrocnemius protein FSR tended to increase with increasing dietary Leu supplementation in ISS+ pigs ($P = 0.085$). LD protein FSR tended to be higher in LEU-M than CON pigs during ISS ($P = 0.072$).

DISCUSSION

The main objective of the current study was to evaluate the effect of dietary Leu supplementation on tissue protein synthesis rates during LPS-induced and short-term ISS; the effects on plasma AA and urea-N levels were also assessed. ISS is characterized by profound alterations in whole-body and tissue protein metabolism, especially in skeletal muscle and in the liver, in order to support the inflammatory response. Leucine is a potent nutritional signal that directly acts on the mTORC1 signaling pathway regulating translation initiation and protein synthesis (Saxton and Sabatini, 2017). Skeletal muscle in particular is sensitive to Leu as an anabolic stimulus, but other tissues can respond as well (Torrazza et al., 2010). The failure of Leu to stimulate protein synthesis in skeletal muscle during ISS has been demonstrated in rodent models of endotoxemia and sepsis (Lang and Frost, 2004, 2005), largely through disruptions in the mTORC1 signaling pathway and changes in AA transporter expression (Kazi et al., 2011; Laufenberg et al., 2014).

We previously established that dietary Leu supplementation resulted in a linear reduction in whole-body protein synthesis and degradation in healthy pigs, but did not affect any aspect of whole-body protein turnover during ISS (Rudar et al., 2016). However, Leu may elicit different responses in visceral and peripheral tissues during ISS. Therefore, rates of tissue protein synthesis in pigs fed Leu above the estimated SID Leu: Lys ratio for PD were determined with a flooding dose of L-[ring- $^2\text{H}_5$]Phe, 36 h after ISS induced by a single intramuscular LPS injection. Based on previous studies, LPS-induced ISS produces a predictable response immediately following the initial LPS injection, while avoiding the potential to develop tolerance to LPS (Rakhshandeh and de Lange, 2012).

Table 1. Ingredient composition and nutrient content of the experimental diets.

Item	CON ¹	LEU-M ²	LEU-H ³
Ingredient, % (as fed)			
Corn starch	42.52	42.56	42.61
Sucrose	20.00	20.00	20.00
Soy protein isolate	21.90	21.90	21.90
Cellulose	4.00	4.00	4.00
Corn oil	4.00	4.00	4.00
Limestone	1.40	1.40	1.40
Dicalcium phosphate	1.53	1.53	1.53
Salt	0.30	0.30	0.30
Magnesium sulfate	0.40	0.40	0.40
Potassium sulfate	0.63	0.63	0.63
Vitamin–mineral mix ⁴	0.60	0.60	0.60
Titanium dioxide	0.20	0.20	0.20
L-Lys-HCl	0.39	0.39	0.39
L-Threonine	0.19	0.19	0.19
DL-Methionine	0.29	0.29	0.29
L-Tryptophan	0.07	0.07	0.07
L-Histidine	0.11	0.11	0.11
L-Valine	0.03	0.03	0.03
L-Leucine	—	0.69	1.37
L-Aspartate	0.73	0.37	—
L-Glutamate	0.73	0.37	—
Calculated nutrient content ⁵			
ME, MJ/kg	15.46	15.46	15.46
CP, %	19.81	19.81	19.81
SID lysine, %	1.36	1.36	1.36
SID leucine, %	1.36	2.04	2.72
SID isoleucine, %	0.82	0.82	0.82
SID valine, %	0.87	0.87	0.87
Crude fat, %	4.03	4.03	4.03
Ca, %	0.78	0.78	0.78
P, %	0.38	0.38	0.38
Na, %	0.37	0.37	0.37
Analyzed nutrient content			
DM, %	94.1	94.1	93.8
CP, %	21.1	20.8	21.2
Total lysine, %	1.40	1.49	1.47
Total leucine, %	1.56	2.42	3.18
Total isoleucine, %	0.91	0.96	0.89
Total valine, %	0.94	1.00	0.93

¹CON = 1.36% standardized ileal digestible (SID) Leu (SID Leu: Lys = 100 [% Lys]).

²LEU-M = 2.04% SID Leu (SID Leu: Lys = 150 [% Lys]).

³LEU-H = 2.72% SID Leu (SID Leu: Lys = 200 [% Lys]).

⁴Supplied per kilogram of complete diet: Vitamin A, 12,000 IU as retinyl acetate; vitamin D₃, 1,000 IU as cholecalciferol; vitamin E, 48 IU as DL- α -tocopherol acetate; vitamin K, 3 mg as menadione; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; vitamin B12, 0.03 mg; biotin, 0.24 mg; Cu, 18 mg from CuSO₄·5H₂O; Fe, 120 mg from FeSO₄; Mn, 24 mg from MnSO₄; Zn, 126 mg from ZnO; Se, 0.36 mg from FeSeO₃; I, 0.6 mg from KI (DSM Nutritional Products Canada Inc., Ayr, ON).

⁵Based on nutrient contents in feed ingredients according to [NRC \(2012\)](#) and nutrient content of soy protein isolate according to the supplier (Archer Daniels Midland Company Inc., Woodstock, ON).

In the current study, and based on 12-h sampling intervals, plasma AA concentrations were largely constant over time in ISS– pigs. Conversely, plasma AA concentrations peaked in ISS+ pigs 12 h after ISS, even though feed intake patterns were

kept identical for ISS– and ISS+ pigs. Among the NEAA, concentrations of Ala and Gln, which are critical AA that support the acute phase response ([Oehler and Roth, 2003](#)), were substantially higher in ISS+ pigs. These results are in agreement with

Figure 1. Plasma total AA (TAA; A), essential AA (EAA; B), nonessential AA (NEAA; C), and branched-chain AA (BCAA; D) concentrations ($\mu\text{mol/L}$) during immune system stimulation (ISS) in ISS $-$ and ISS $+$ pigs fed increasing levels of dietary standardized ileal digestible (SID) Leu above the estimated SID Leu: Lys ratio for whole-body protein deposition. Values are least-squares means $\pm$ SE (largest value selected); CON = 1.36% SID Leu (SID Leu: Lys = 100 [% Lys]); LEU-M = 2.04% SID Leu (SID Leu: Lys = 150); LEU-H = 2.72% SID Leu (SID Leu: Lys = 200). ISS was induced with a single intramuscular injection of *Escherichia coli* lipopolysaccharide (ISS $+$) at 0 h; ISS $-$ pigs were administered sterile saline. There is a significant interaction between treatment and time during ISS for TAA, EAA, NEAA, and BCAA concentrations ($P < 0.05$). * $P < 0.05$ for the linear effect of increasing SID Leu: Lys on AA concentrations in ISS $+$ pigs at 36 h.

Bruins et al. (2003) who demonstrated that arterial plasma concentrations of Ala and Gln, and the release of Ala and Gln from skeletal muscle, were increased in pigs during endotoxemia. Conversely, Garcia-Martinez et al. (1993) reported a specific decrease in the blood concentrations of most gluconeogenic AA in rats starting 2 h after LPS administration. This discrepancy may be due to a rapid increase in whole-body utilization of these AA before muscle protein degradation is increased in order to increase the supply of these AA to visceral tissues (Garcia-Martinez et al., 1993). In the current study, the observed pattern of plasma AA concentrations over time indicates that substantial modifications in tissue protein metabolism occurred within 12 h following ISS. It is likely that the largest ISS-associated changes in tissue protein synthesis occurred at this time and when feed intake was minimal, rather than at 36 h when tissue

protein synthesis rates were measured. Given the low level of feed intake at 12 h following ISS, anticipated responses in tissue protein synthesis would have been confounded with the low level of Leu intake. However, PD was lower (Rudar et al., 2016) and PUN was higher 36 h after ISS was induced, suggesting prolonged alterations in protein and AA metabolism during ISS in this study.

The negative linear effect of dietary Leu supplementation on plasma Ile and Val, but not Leu, concentrations in ISS $+$ pigs at 36 h implies a specific increase in Leu utilization in LEU-H pigs. Although the exact fate of Leu in this study could not be determined, Leu is an essential nitrogen donor for Ala and Gln synthesis in skeletal muscle (Ruderman and Berger, 1974). In postabsorptive men, the transfer of Leu-N to the Gln α -amino group accounted for 21% of Leu-N flux (Darmaun and Dechelotte, 1991). Moreover, LPS increases

Figure 2. Plasma urea nitrogen (PUN) concentrations (mg/dL) during immune system stimulation (ISS) in ISS- and ISS+ pigs fed increasing levels of dietary standardized ileal digestible (SID) Leu above the estimated SID Leu: Lys ratio for whole-body protein deposition. Values are least-squares means $\pm$ SE (largest value selected); CON = 1.36% SID Leu (SID Leu: Lys = 100 [% Lys]); LEU-M = 2.04% SID Leu (SID Leu: Lys = 150); LEU-H = 2.72% SID Leu (SID Leu: Lys = 200). ISS was induced with a single intramuscular injection of *Escherichia coli* lipopolysaccharide (ISS+) at 0 h; ISS- pigs were administered sterile saline. There is a significant interaction between treatment and time during ISS for PUN ($P < 0.05$). * $P < 0.05$ for the linear effect of increasing SID Leu: Lys on PUN in ISS+ pigs at 36 h.

Table 2. Tissue protein fractional synthesis rate (FSR) during immune system stimulation (ISS) in ISS- and ISS+ pigs fed increasing levels of dietary standardized ileal digestible (SID) Leu above the estimated SID Leu: Lys ratio for whole-body protein deposition¹

Tissue	Treatment ²				SE	P-value	
	CON ISS-	CON ISS+	LEU-M ISS+	LEU-H ISS+		ISS ³	Linear ⁴
Number of animals	6	7	8	7			
FSR, %/d							
Plasma	35.9	34.3	34.5	32.1	2.6	0.643	0.506
Liver	83.0	96.2	93.3	84.9	4.2	0.031	0.052
Gastrocnemius	4.32	3.94	4.18	5.17	0.52	0.603	0.085
LD	5.50	4.45	6.46	4.73	0.61	0.221	0.725

¹Values are least-squares means $\pm$ SE (largest value selected).

²CON = 1.36% SID Leu (SID Leu: Lys = 100 [% Lys]); LEU-M = 2.04% SID Leu (SID Leu: Lys = 150); LEU-H = 2.72% SID Leu (SID Leu: Lys = 200); ISS-: single intramuscular injection of sterile saline; ISS+: single intramuscular injection of *Escherichia coli* lipopolysaccharide.

³P-value for the contrast between ISS- and ISS+ pigs fed CON.

⁴P-value for the linear effect of increasing SID Leu: Lys in ISS+ pigs on mean isotopic enrichment of Phe in free and protein-bound pools or tissue protein FSR.

muscle Gln release and Gln synthetase activity, with no change in glutaminase activity, in rats (Austgen et al., 1992), which suggests that the rate of N transfer from Leu to Gln would increase during ISS.

In the current study, there was no significant effect of ISS on gastrocnemius or LD protein FSR. This observation contrasts with previous studies in pigs and rats that demonstrate substantial reductions in skeletal muscle protein synthesis in response to ISS, induced by either LPS (Orellana

et al., 2002; Lang and Frost, 2004), sepsis (Breuille et al., 1998; Lang and Frost, 2005), or pro-inflammatory cytokines (Lang et al., 2002). Despite an initial decline during the acute phase of sepsis, a normal rate of gastrocnemius protein synthesis was restored in rats during the chronic phase (Breuille et al., 1998). However, gastrocnemius protein mass continued to decline, which indicates a prolonged elevation in the rate of skeletal muscle protein degradation. Based on the observed changes in plasma

AA concentrations, it is possible that changes in muscle protein FSR are already diminished at 36 h after ISS was induced. Skeletal muscle PD contributes to at least 50% of whole-body PD in pigs (Mahan and Shields, 1998), and whole-body PD in ISS+ pigs was lower during the challenge period (Rudar et al., 2016). Therefore, the observed reduction in whole-body PD in part may be due to greater skeletal muscle protein degradation, assuming that any decrease in skeletal muscle protein synthesis was transient.

Studies using different models of ISS have shown that hepatic protein synthesis is generally increased during ISS, accounting for an increase in the synthesis rate of both structural and secreted proteins (Vary and Kimball, 1992). In the current study, we observed a 16% increase in liver protein FSR in ISS+ pigs with no corresponding increase in plasma protein FSR. The observed rates of liver and plasma protein synthesis are similar to rates reported previously in pigs (Bregendahl et al., 2004, 2008), and support the key role of the liver in whole-body protein metabolism during ISS. Conversely, Litvak et al. (2013) reported no effect of ISS on liver protein FSR despite an increase in plasma protein FSR during prolonged ISS. Albumin, which forms the largest proportion of plasma protein, is a negative APP in pigs. A substantial reduction in plasma albumin concentration may therefore negate the contribution of an ISS-induced increase in the isotopic enrichment of albumin to total plasma protein. At the same time, the concentration of other APP (e.g., fibrinogen and haptoglobin) may not have increased to the extent observed by Litvak et al. (2013).

Inflammation-induced anabolic resistance to Leu in skeletal muscle is largely mediated through alterations in the mTORC1 signaling pathway (Lang and Frost, 2005; Kazi et al., 2011). In the adult rat, skeletal muscle remains resistant to Leu during acute endotoxemia and sepsis (Lang and Frost, 2004, 2005; Laufenberg et al., 2014), but some sensitivity is recovered during prolonged sepsis (Vary, 2007). This suggests that the mechanisms of ISS-induced anabolic resistance may vary depending on the disease model and physiological state of the animal. However, there is limited information available on the ability of Leu to regulate tissue protein synthesis past the short-term metabolic response characteristic of acute endotoxemia. For these reasons, we anticipated that dietary Leu supplementation may increase skeletal muscle protein synthesis while decreasing liver protein synthesis in starter pigs during ISS. In the current study, there was a linear increase in gastrocnemius protein FSR

with dietary Leu supplementation, and a marginal increase in LD protein FSR from CON to LEU-M pigs, during ISS. This result suggests two distinct possibilities. First, resistance of skeletal muscle to Leu is transient and does not persist longer than 36 h. Second, skeletal muscle is still resistant to Leu, but the amount of dietary Leu supplied to LEU-M and LEU-H pigs exceeded a threshold needed to elicit a response in protein synthesis. Since gastrocnemius and LD protein FSR were not reduced in ISS+ pigs, it is more likely that skeletal muscle resistance to Leu was transient. Skeletal muscle in the newborn pig is sensitive to AA and Leu during acute endotoxemia, which supports the possibility that Leu may improve skeletal muscle protein synthesis during ISS (Orellana et al., 2007; Hernandez-Garcia et al., 2016). Leucine also appears to reduce MuRF1 expression and LC3-2: total LC3 ratio, which is associated with protein degradation by the ubiquitin–proteasome and autophagy–lysosome pathways, respectively, in neonatal pigs during acute endotoxemia (Hernandez-Garcia et al., 2016). However, gastrocnemius and LD protein synthesis were not determined before ISS was induced, and we are therefore unable to conclude that skeletal muscle protein was fully sensitive to Leu.

In the current study, liver protein FSR tended to decline with dietary Leu supplementation in ISS+ pigs. In newborn pigs, liver protein synthesis is largely unaffected by dietary Leu supplementation (Torrazza et al., 2010; Columbus et al., 2015) and insulin (O'Connor et al., 2003). Despite increasing skeletal muscle protein synthesis, AA administration did not further augment the ISS-induced increase in liver protein synthesis in newborn pigs (Orellana et al., 2007). These results suggest that the liver is insensitive to Leu-induced anabolism and that Leu may in fact suppress liver protein synthesis during ISS. Since plasma protein FSR was not affected by dietary Leu supplementation, it appears that the ability of the pig to respond to an inflammatory insult through the synthesis of APP is not blunted. Instead, the lower rate of liver protein synthesis may indicate increased AA availability for skeletal muscle protein synthesis and PD. Considering that the regulation of mTORC1 signaling differs between skeletal muscle and liver protein during ISS (Kimball et al., 2003), it is possible that Leu elicits distinct responses in these tissues. The apparent increase in skeletal muscle protein synthesis and decrease in liver muscle protein suggests that dietary Leu supplementation may support repartitioning of AA from visceral to peripheral PD during ISS.

The results of the current study show that ISS resulted in acute increases in plasma AA concentrations that likely reflect changes in tissue protein synthesis and degradation. However, long-term changes in plasma BCAA concentrations may be due to Leu-induced elevated rates of BCAA catabolism, which is further exacerbated by ISS. Although no reduction in gastrocnemius and LD protein synthesis was observed, liver protein synthesis tended to increase 36 h after ISS. Dietary Leu supplementation appeared to exploit the differential regulation of skeletal muscle and liver protein synthesis during ISS. The reduction in liver protein synthesis and increase in gastrocnemius muscle protein synthesis in response to dietary Leu supplementation may facilitate AA utilization in skeletal muscle for PD, but the effect of Leu on protein degradation in these tissues should also be considered. The optimum SID Leu: Lys ratio to minimize visceral PD and maximize skeletal muscle PD during ISS should be determined in order to optimize AA nutrition for pigs.

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