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Whole body and splanchnic amino acid metabolism in sheep during an acute endotoxin challenge

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Abstract

Supplemented protein or specific amino acids (AA) are proposed to help animals combat infection and inflammation. The current study investigates whole body and splanchnic tissue metabolism in response to a lipopolysaccharide (LPS) challenge with or without a supplement of six AA (cysteine, glutamine, methionine, proline, serine and threonine). Eight sheep were surgically prepared with vascular catheters across the gut and liver. On two occasions 4 sheep were infused through the jugular vein for 20 h with either saline or LPS from E.coli (2 ng/kg BW/min) in a random order plus saline into the mesenteric vein; the other 4 sheep were treated with saline or LPS via the jugular vein plus saline or 6 AA infused into the mesenteric vein. Whole body AA irreversible loss rate (ILR) and tissue protein metabolism were monitored by infusion of [ring-$^{2}H_{2}$]phenylalanine. LPS increased ($P<0.001$) ILR (+17%,), total plasma protein synthesis (+14%) and lymphocyte protein synthesis (+386%) but decreased albumin synthesis (-53%, $P=0.001$) with no effect of AA infusion. Absorption of dietary AA was not reduced by LPS, except for glutamine. LPS increased hepatic removal of leucine, lysine, glutamine and proline. Absolute hepatic extraction of supplemented AA increased but, except for glutamine, this was less than the amount infused. This increased net appearance across the splanchnic bed restored arterial concentrations of five AA to, or above, values for the saline-infused period. Infusion of key AA does not appear to alter the acute period of endotoxaemic response, but may have benefits for the chronic or recovery phases.

Abbreviations: AA, amino acids; DM dry matter; FSR, factional synthesis rate; ILR, irreversible loss rate; LPS, lipopolysaccharide; PDV, portal-drained viscera; REML, residual maximum likelihood

Introduction

Infection or inflammation cause marked responses in amino acid (AA) and protein metabolism. These include alterations in plasma AA concentrations, with many decreased[1-3], plus accompanying changes in whole body AA irreversible loss rates (ILR)[1,3,4]. These responses probably reflect complex interactions between the immune system and key regulatory organs. For example, lowered AA plasma concentrations may result from reduced net absorption, either from inhibition of intake that accompanies inflammation and sepsis[5], or increased oxidation by the portal-drained viscera (PDV), as observed with certain gastro-intestinal infections[6]. Alternatively, demands within specific tissues can increase removal of AA from the blood circulation to support synthesis of either additional proteins or specific metabolites. For example, manufacture of positive acute phase proteins increases liver utilisation of essential AA[4], especially phenylalanine[7], while elevated hepatic glucose synthesis during major stress[8] elevates metabolism of glucogenic AA, although this is not always observed[4]. Similarly, production of additional glutathione to provide anti-oxidant protection adjacent to sites of inflammation and pro-oxidant activity can alter demand for cysteine[9]. Furthermore, activation of the immune system elevates net use of AA to support proliferative responses associated with infection or surgery[10], and also increase hepatic use of glutamine[4], a known regulator of intermediary metabolism[11]. While some of these needs are general and require most AA, as in the case of cellular proliferation, other reactions will be restricted to one, or just a few, AA and this will leave the remainder in disproportionate excess and lead to their disposal as urea and lead to the net nitrogen losses characteristic of inflammation and sepsis[12].

Reduced plasma AA during infection or inflammation can be offset by either additional protein or AA supply[13] but the quantities required differ between AA[3] and between type and magnitude of the challenge[2]. The effectiveness of such approaches has been demonstrated in septic rodents, where a cocktail of AA reduces the severity of the challenge and enhances the rate of recovery[14]. Future nutritional strategies to help combat the deleterious effects of infection and inflammation and aid recovery require knowledge of both the absolute demands for specific AA[3], and where in the body these requirements arise. The effectiveness of targeted intervention in sheep, based on previous kinetic quantification of AA demands during the acute phase of an inflammatory challenge[3], is addressed in the current study. The focus is on splanchnic tissue metabolism, with consequences on absorption, liver-related protein metabolism and net AA supply to peripheral tissues. This was tested with a cocktail of six AA, based on information gained from an earlier study[3]. Three (methionine, serine and threonine) were chosen because the ILR through plasma decreased markedly in response to LPS[3], suggestive of a deficient supply during endotoxaemia. Cysteine has been reported as beneficial for septic rats[14] and LPS-challenged pigs[15] but showed no change in ILR in the previous sheep study[3]. Also included was proline, which showed similar responses cysteine with no effects on ILR but marked decreases in plasma concentration under an LPS challenge[3]. The final AA in the cocktail was glutamine, supplementation of which is often used in clinical situations[16] and exhibits increased turnover during cancer[17] and endotoxin challenge[3]. For all these AA, the amounts infused were based on the product of their ILR and the fractional reduction in plasma concentration as observed under similar experimental conditions previously[3].

Materials and Methods

Sheep and diets

All procedures were approved by the Ethical Review Committee of the Rowett Institute of Nutrition and Health and conformed to UK legislation under the Animals (Scientific Procedures) Act 1986. Suffolk cross lambs (n=8, 2 females, 6 castrate males; 12-16 months old, 37-54 kg live weight) were prepared with silicone rubber catheters in the aorta, mesenteric vein, portal vein and hepatic vein[18]. During the 2 week recovery period from surgery the sheep offered a mixed roughage–concentrate diet with the following composition, g/kg as fed (2 x 500 g/d as fed: hay 500, barley 300, molasses 100, fishmeal 90, vitamin and mineral mix 10; 830 g DM/kg; 21.3 gN/kg DM, 11.0 MJ metabolisable energy/kg DM) at an estimated 1.0-1.3 x energy maintenance based on metabolic BW ($kg^{0.75}$). Subsequently they were acclimatised over 1 week to metabolism crates, with the daily feed provided as 24 hourly portions, and then allocated to treatments when a temporary polyvinyl catheter was inserted into the jugular vein[18].

At treatment allocation, the sheep (n=8) were divided between two groups, balanced for gender and weight. Within each group the sheep were measured on two experimental days, 7 days apart. For Group A (n=4) the two infusion days involved either sterile 0.15 M sodium chloride (control) infused into both the jugular vein (15 g/h) and mesenteric vein (40 g/h) for 20 h while for the other experimental day the jugular vein infusion (15g/d) involved lipopolysaccharide (LPS; from *E. coli* 155, serotype 055:B5, 2 ng/min per kg BW) as described previously[3]. For Group B (n=4), one experimental day was identical to the control procedure used for Group A while the other experimental day involved a jugular vein infusion of LPS, identical to Group A, plus a sterile mixture of AA into the mesenteric vein (40 g/h). The order of infusions (saline versus LPS, with or without AA) was randomised between sheep. Therefore, all 8 sheep received a control (saline) jugular infusion, while 4 received LPS as a treatment and 4 received LPS plus AA. The amounts for each of the AA infused were calculated from the product of their ILR and the fractional decrease in arterial concentration from control value in response to a similar dose of LPS, both as reported previously[3]. For sheep with a body weight of 50 kg, the concentrations of AA-N in the supplement were cysteine (68 mM), glutamine (320 mM), methionine (38 mM), proline (64 mM), serine (124 mM) and threonine (138 mM) dissolved in 0.15 M sodium chloride. For sheep of other weight the concentration of the infusate was adjusted based on $BW^{0.75}$.

Just prior to the start of each 20 h infusion period a background blood sample was taken for evaluation of clinical parameters (including blood haemoglobin, plasma albumin, white cell count and cell type distribution) and determination of blood and plasma dry matter (by freeze-drying, each in triplicate). In order to measure plasma flow across the splanchnic tissues sterile sodium p-aminohippurate (0.1 M prepared in 0.05 M sodium phosphate buffer pH 7.4), and containing 250 I.U./g of heparin (Leo Laboratories, Princes Risborough, Bucks), was also infused at a rate of 40 g/h into the mesenteric vein over the period 15-20 h. This was mixed with the appropriate AA or 0.15 M sodium chloride infusate via a t-piece connector. From 12 to 20 h, a solution of 15 mM $[^{2}H_{5}]$phenylalanine (99 atom %; Cambridge Isotope Laboratories, Andover, MA, USA) in 0.15 M sterile saline was infused at 10 g/h into the jugular vein, again via a t-piece connector.

Between 16 to 20 h of LPS-infusion, blood was withdrawn continuously over iced-water as 4 x 1h samples from the arterial, portal and hepatic venous catheters[18] with 12 ml taken for each collection. At 12 and 20 h, 20 ml of arterial blood was withdrawn and maintained at room temperature for immediate processing of lymphocytes. At 20 h, the various infusions were stopped and 6 g of a sterile saline containing 24 mg of Evan's Blue injected via the jugular catheter. Then 2.5 ml of blood were withdrawn at 3, 6, 9 and 12 min after injection to allow estimation of plasma volume[19].

Chemical analyses

Clinical blood parameters were determined as described previously[3]. Immediately following each hourly collection, blood analysis (pH, pO_2, pCO_2 and haemoglobin) was performed with an ABL650 Blood Gas Analyser (Radiometer, Copenhagen, Denmark) and packed cell volume determined by micro-centrifuge. Blood samples were then centrifuged at 1000 *g* for 15 min and the plasma used for various analyses using gravimetric procedures[18]. All blood samples taken were analysed individually, parameters were then calculated separately for each hour of collection and then the mean of these used for statistical analysis.

The p-aminohippurate concentration was quantified on 0.7g plasma[18] while 1g plasma from each sample site was retained for enrichment analysis of [$^{2}H_5$]phenylalanine. To another 1.4 g plasma was added 0.6 g of a mixture of [U-^{13}C] algal hydrolysate containing [5-^{15}N]glutamine, [indole-^{15}N]tryptophan, [1-^{13}C]cysteine and [$^{15}N_2$]urea to allow determination of AA concentrations by isotope dilution[20,21]. This sample was divided into two equal portions with one kept in reserve. A further portion of fresh plasma (0.4 g) was analysed with commercial kits for total protein, albumin, glucose (Thermo Scientific; kits 981-387, 981-767, 981-304, respectively), ammonia (Sentinel Diagnostic; kit 17660) and lactate (Trinity Biochemicals; kit 735-10) on a clinical analyser (Kone Limited). Plasma albumin was isolated from arterial plasma[22]. Concentrations of Evans Blue bound to plasma protein were determined spectrophotometrically[19] and values extrapolated to zero time injection to allow estimation of plasma volume. Determination of the enrichments of plasma free [$^{2}H_5$]phenylalanine and the extraction, hydrolysis and analysis of both albumin and plasma total protein labelled with [$^{2}H_5$]phenylalanine were as described previously[22]. Lymphocytes were isolated from the 20 ml blood samples taken at the start and end of the isotope infusion. The blood was first gently diluted 1:1 with 0.15 M NaCl and 5 ml portions slowly layered onto 5 ml Histopaque 1077 lymphocyte separation media (Sigma Bioscience), in 8 separate tubes, with care taken to avoid mixing of the two layers. The tubes were then centrifuged at 700 g for 20 minutes at 20^{o}C with no brake applied. The lymphocyte layer at the interface was then carefully removed by Pasteur pipette from each tube and these all transferred to a 10 ml glass hydrolysis tube and diluted to 10 ml with ice-cold 0.15 M NaCl saline. This solution was centrifuged at 1500 g for 15 min at 4° C and the pellet re-suspended in 4 ml of ice-cold lysis buffer (9:1 (vol:vol) ammonium chloride (8.3 g/l): 0.17 M Tris-HCl buffer pH 7.65). This was re-centrifuged at1500 g for 15 min at 4°C and the supernatant containing the contents from red blood cell lysis decanted. The pellet was then washed with ice-cold saline and re-centrifuged on three more occasions. After the final wash the pellet was re-suspended in 5 ml of distilled water and stored frozen at –20°C until further analysis. Lymphocyte protein was prepared in a manner similar to the general procedure for albumin and total plasma protein and involved thawing the stored suspension and deproteinisation with addition of 0.8 ml of 48 % (w/v) sulphosalicylic acid (SSA). After standing on ice for 10 min the sample was centrifuged at 1500 g for 20 minutes at 4 °C. The sample was then washed twice with 8 ml 8 % ice cold SSA and centrifuged on each occasion. The supernatant was removed and to the pellet a phenol crystal was added (to protect aromatic AA against oxidation) followed by 4 ml of 4 M HCl. This was then hydrolysed for 18 h at 105°C in a heating block. Subsequent steps were then as described previously[22].

Calculations

Plasma flows (kg/min) were determined by a gravimetric approach[18] with blood flow calculated from plasma flow/(1-packed cell volume). Hepatic artery (A) flow (blood or plasma) was determined as the difference between the flows in the hepatic vein (FH) and hepatic portal vein (FP). The plasma and blood water flows were calculated from the dry matters in order to quantify urea transfers[23]. In general, net mass transfers (µmol/min) of individual AA or metabolites across the PDV were calculated as:

$$(C_h \times FH) - (C_p \times FP) - (C_a \times FA)$$

where C_a, C_p and C_h are metabolite concentrations (µmol/kg) in arterial, hepatic portal vein and hepatic vein fluids (blood for oxygen, ammonia; blood water for urea; plasma for all other measurements).

For protein synthesis (µmol/min) estimates based on isotope transfers across the gastro-intestinal tract and liver the appropriate calculations were:
across the portal drained viscera (PDV)

$$(C_p \times E_p - C_a \times E_a) \times FP / E_x$$

and across the liver:

$$\frac{(C_b \times E_b \times FH) - (C_p \times E_p \times FP) - (C_a \times E_a \times FA)}{E_x}$$

where E_h, E_p and E_a, are the respective enrichments (molar % excess) of free [$^2H_5$]phenylalanine in plasma from the hepatic vein, portal vein and artery, and where E_x is the enrichment value selected as representative of the precursor pool. For comparison with whole body irreversible loss rate measurements then E_x was based on the arterial value, but for other comparisons E_x was assumed similar to hepatic venous enrichments as this has been shown to reflect well values for export proteins[22]. Both estimates of precursors, however, are less than values observed in the intracellular pools of either the liver[22] or the gastro-intestinal tract[24]. For both net transfer data and isotope kinetics the concentrations, enrichments and flows were calculated for each individual hour of collection and then averaged.

Whole body irreversible loss rate (WB ILR, mmol/h) of tracee were estimated by the standard procedure i.e.

$= (99/E_a - 1) \times$ infusion rate (mmol/h)

where 99 is the enrichment (molar % excess) of the [$^2H_5$]phenylalanine infusate.

Enrichments for both total protein and albumin from plasma altered in a linear manner over the times of collection and their respective gradients (change molar % excess/h) were divided by E_h (representative of the precursor for export proteins[22]) and adjusted to give daily fractional synthesis rates (FSR). These FSR were then converted to absolute synthesis rates (g/d) by:

= FSR/100 × plasma concentration (g/l) × plasma volume (l)

Where total plasma volume was determined by dilution of Evans Blue[19]. For lymphocytes, a value of 28.2 pg protein/cell was adopted[25].

Power calculations and statistical analyses

The number of sheep per treatment group (control combined with LPS or control combined with LPS+AA) was based on comparing the effect of LPS+AA to the effect of LPS alone. The power calculations were performed at a power of 80% for the 5% significance level, with the between-sheep variance obtained from previous studies[3,22]. A considerable number of parameters were to be assessed in this study but the decision on number of sheep per treatment group was based on the following two outcomes that were deemed most indicative of the restorative effect of AA supplementation on an LPS challenge. The first parameter involved restoration of the arterial concentrations for the 6 AA infused in combination with LPS to at least 90% of the control (saline) values. Expected decreases for these six AA were expected to be 23 to 73% by the action of LPS alone[3] with between-sheep SD 13-43% of control values[3], yielding n per group=4. The second parameter involved changes fractional synthesis rate for plasma albumin, a negative acute phase protein, and based on an SD of 0.49 %/d[22], a change of 1.4%/d, equivalent to the difference between fed and fasted sheep[22], would be observed with n per group=4. Additional power was gained by initial selection of 6 sheep per treatment group. Unfortunately, one sheep required to be euthanised during the surgical procedure, while three others developed either non-patent catheters in either the portal or hepatic veins before the end of the study. Thus 11 sheep had measurements reliant on arterial samples (this included the two criteria used for the power calculations above) but only 4 sheep per treatment group for the full splanchnic transfers. In practice the changes based on arterial values only were similar between the 11 and 8 sheep comparisons. Therefore, for the main text, data are presented for the 8 sheep that had the full working splanchnic-bed catheters. Of these, four received a saline infusion or LPS infusion on two separate days. The other four sheep received a saline infusion or LPS+AA infusion on two separate days. The order of infusions was randomised within each group. Although this is small study, the use of a frequent feeding regimen, continuous administration of low dose of LPS over the experimental duration and collection of integrated blood samples all helped reduce associated variance and meant that fewer animals were needed to detect statistical differences. In addition, where appropriate, results from the 11 sheep are presented as online Supplementary material.

Data were analysed as a mixed model using the residual maximum likelihood (REML) estimation procedure in Genstat 13th edition, release 13.2 (VSN International, Hemel Hempstead, UK). The influence of LPS (independent of whether AA were infused or not) was assessed with sheep and period (experimental day) within sheep as random effects while period and LPS status (present or absent) and their interaction were regarded as fixed effects. To assess the influence of AA infusion, sheep and period within sheep were considered as random effects while period and treatment and their interaction were considered as fixed effects, where treatment involved saline, LPS and LPS + AA infusions.

All data are presented as predicted means from the REML analysis, with the maximum SED value also given for the various comparisons. $P<0.05$ was taken as evidence of a significant response and $0.05<P<0.10$ as weak evidence

Results

Whole body responses and arterial concentration changes

Within the first 4 h of LPS infusion there were increases in body temperature (1.0-2.0°C) and respiration rate, as observed previously[(3)]. During the same period, occasionally there were mild reluctances to eat but this never exceeded more than two hourly meals, and these was eaten soon thereafter so for the total 20 h there were no refusals. Over the period of blood collection (16-20 h after the start of LPS or vehicle infusion), endotoxaemia did not affect either arterial blood pH or haemoglobin status (data not shown). Haemoglobin was lower during the second period (10.7 v. 9.1 SED 0.61, P = 0.027), as was plasma albumin (27.1 v 26.5 g/l, SED 0.22, P = 0.013), both possibly related to the amount of blood withdrawn over the various procedures. Blood white cell numbers were slightly increased just prior to LPS infusion for the second period (10.24 v. 12.25 x 10^9 cells/l blood, SED 0.870, P = 0.046). Most of this could be attributed to whether the sheep had received LPS or saline during the first period (respectively 14.63 v. 11.35 x 10^9 cells/l blood, SED 1.487, P = 0.055). There were no differences between allocated groups (saline or LPS) in white cell count prior to period 1. There were no period or previous treatment effects on the proportion of neutrophils (34%) and lymphocytes (64%) in the monocyte population prior to each infusion period. At the end of the 20 h infusion, numbers of lymphocyte cells had more than doubled in response to LPS challenge compared with saline infusion (Table 1).

Endotoxaemia tended to cause a 30% decrease in arterial glucose (P = 0.067), while lactate concentrations were unaffected (Table 1). Arterial plasma concentrations of both total protein (-6%, P = 0.018) and albumin (-4%, P = 0.007; Table 1) were reduced by LPS. In contrast, while the FSR of albumin was also decreased (-48%, P < 0.001) that of total protein was increased (+37%, P 0.012; Table 2). As total plasma volume (average 2.18 l) was unaffected by either period or treatment, then similar directions of change were also observed for the ASR of both albumin and total protein (both $P < 0.01$; Table 2) in response to LPS. The substantial increase in lymphocyte numbers was accompanied by a 63% increase in FSR ($P = 0.043$ Table 2), while the ASR was elevated by 386% ($P< 0.001$). For all these variables, the responses to LPS were independent of whether the supplemental AA were provided or not. Responses reported in both Table 1 and 2 for the 8 sheep with complete functional catheters across splanchnic tissues at the end of the study were similar to data obtained from the original 11 sheep (online Supplementary Tables S1 and S2).

Arterial plasma concentrations of AA

For most non-infused AA, the arterial plasma concentrations were reduced substantially with both LPS and LPS+AA treatments (by 20-50%, $P < 0.01$; Table 3). The exceptions were tryptophan, where only a tendency was observed (-15%, $P = 0.079$), while for phenylalanine the plasma concentration increased (+30%, $P < 0.001$). The reductions for the non-infused AA were not influenced by the infusion of the six AA. LPS infusion also increased plasma urea (22%, P =0.014).

For the 6 AA that formed the supplement, these also showed decreased plasma concentrations when the sheep were challenged with LPS but infused with saline (reduced by 14-74%, all $P < 0.05$). For the four sheep where the supplement was infused this restored plasma concentrations to saline-infused values for cysteine, glutamine, methionine, proline and serine (Table 3). The infusion of threonine over-compensated (P=0.007).

Splanchnic bed metabolism

Plasma flows in the portal vein, hepatic vein and hepatic artery (means 1.347, 1.428 and 0.080 kg/min respectively) were not altered by the endotoxin challenge (data not shown). Neither were there any effects of LPS (with or without supplemental AA) on splanchnic tissue oxygen uptake (-112 and –113 μmol/min for PDV and liver, respectively). Although glucose concentrations were higher in the hepatic vein than either the portal vein or hepatic artery (3.81, 3.56, 3.51 mM, respectively, SED 0.041, P<0.001) with, in consequence, more glucose appearance across the liver than the PDV (+360 vs + 89 μmol/min, SED 79.1, P<0.001), there was no effect of LPS infusion, with or without infused AA present. Similarly, lactate transfers were also unaffected by endotoxaemia (-130 and +64 μmol/min for PDV and liver, respectively).

PDV AA transfers

All AA, except glutamine, showed net positive appearances (uptake) across the PDV under the various experimental conditions (Table 4). Over the 4 h of sampling, infusion of LPS did not alter net PDV appearance, compatible with the lack of effect of the endotoxin on food intake. In contrast, the net PDV appearance of glutamine across the PDV was negative during saline infusions (indicative of metabolism of endogenous glutamine by the gut tissues) but this was reduced by 67% (P=0.014) with LPS infusion (without supplemental AA). Neither removal of urea nor appearance of ammonia across the PDV was affected by LPS infusion. As expected, mesenteric vein infusion of the six AA increased their portal vein appearance (all P < 0.001). For the 6 infused AA, apparent recovery within sheep across the PDV were not significantly different from 100% for methionine, proline, serine, threonine and glutamine, but only 71% for cysteine (P=0.004). The amount of glutamine infused was sufficient to provide a net positive supply to the liver (Table 4). PDV appearances of alanine and lysine (both P < 0.05) were also increased by infusion of the AA supplement. For alanine this restored values to those of the saline-infused period, while for arginine net supply increased above that for the control (saline) period (P =0.012).

Hepatic AA transfers

Except for glutamine and glutamate, during saline infusion there was net removal of each AA across the liver and these were different from zero (P<0.05) except for aspartate and cysteine. During saline infusion, there was net hepatic export of glutamate (P<0.001). LPS infusion (without supplemental AA) increased net hepatic removal (P<0.05) of 6 AA (Table 5) and these contributed to the 75% extra extraction of total AA-N (P = 0.012). For the non-infused AA, hepatic removal was not affected by the supplemental AA in the presence of LPS (Table 5). In contrast, extraction by the liver of all the infused AA was markedly increased (P<0.01) and this resulted in an additional 40% removal of total AA-N (P=0.030).

Net splanchnic AA transfers

The difference between net absorption (plus any infused AA) and hepatic removal represents net splanchnic flow to peripheral tissues (Table 6). With saline infusion, only glycine and glutamine had significant ($P<0.05$) negative net transfers i.e. hepatic uptake exceeded PDV absorption and thus additional amounts were removed from the peripheral circulation. In terms of positive net post-splanchnic supply, most AA had values that were significantly different from zero ($P<0.05$), the exceptions were alanine, cysteine, histidine, phenylalanine, tryptophan and tyrosine. In response to LPS alone, net splanchnic supply decreased for alanine and arginine ($P < 0.05$). Provision of supplemental AA increased net splanchnic supply for cysteine, methionine, proline, threonine (all $P<0.010$) and serine ($P=0.037$), but not glutamine.

Isotope transfers

Phenylalanine whole body ILR was increased in response to LPS infusion, both with and without the AA supplement (mean 17%, $P < 0.001$; Table 7). Endotoxaemia did not alter ILR across the PDV, regardless of whether the arterial or venous plasma enrichment was chosen as precursor (latter data not shown). In contrast, with either method of calculation, both liver ($P = 0.023$) and total splanchnic ($P = 0.034$) metabolism was increased by LPS. In combination, LPS plus supplemental AA increased hepatic protein synthesis by 12% ($P=0.036$) compared with LPS alone. Together, the hepatic and PDV response accounted for approximately 40% of whole body ILR under both saline and LPS infusions, but with the contribution from the liver more than 2-fold that from the PDV.

DISCUSSION

Although experimentally-induced endotoxaemia has proved a popular research tool to study the metabolic events and beneficial nutrition interventions related to inflammation, the responses observed can vary due to many factors, including species, severity of dose and period of measurement. For example, in both pigs[4] and sheep[3] LPS causes a decrease in plasma concentration for most AA. In contrast, in rodents concentrations can increase[26], while in humans both null[27] or decreased[28] responses have been reported. The dose employed in the current study is below that usually adopted, for example only 4% of the hourly dose used in pigs[4] but does give the advantage that pyrexia and anorexia responses are mild and of limited duration yet with similar responses in arterial AA concentrations and metabolic flows[3].

Whole body and splanchnic tissue response to endotoxin challenge

The LPS dose increased whole body ILR of plasma phenylalanine in the sheep, as observed previously[3], in support of observations in pigs[1] and humans[27]. Similarly, the increase in lymphocyte cell numbers is in agreement with other findings during infection and inflammation challenges[3,29], although in shorter-term endotoxaemia studies (< 2.5 h) in human such changes are not observed[30]. Increased cell numbers may involve mobilisation of lymphocytes from pre-existing stores and/or a higher fractional rate of synthesis and the current data confirm that the latter occurred. Nonetheless, the 4-fold increase in total protein synthesis of lymphocytes represented < 0.1% of whole body protein synthesis and would only require <0.2% of the phenylalanine absorbed from the diet. In contrast, the net increase in total plasma protein synthesis (4 g/d, from Table 2) would require an additional 5% of the absorbed phenylalanine. The latter value compares with estimates for the total immune system of approximately 8% in humans[31] and with nutritional costs increased from 1.2 to 6.7% between saline-infused and LPS-challenged chicks[32]. Other components of the immune system, not monitored in the current study and including the thymus, spleen, bone marrow and immune cells, may also be activated[33] with a possible greater contribution from secondary lymphoid organs compared with primary tissues plus blood lymphocytes[29].

Although endotoxaemia is associated with changes in NO status[26], blood (and plasma) flows across the splanchnic tissues remained unaltered. Again this is in line with observations in both pigs[4,26] and rodents[26]. In humans, a near doubling of splanchnic blood flow was observed[27] but this had disappeared 6 h after the bolus injection of endotoxin. LPS also exerts direct effects on the gut, with consequent damage[34] and altered permeability[15]. It is clear, however, that there was no impact on net absorption of most AA in the current experiment but this may relate to the parenteral route of LPS infusion and the low dose employed. Therefore, the altered arterial AA concentrations must be a consequence of altered post-intestinal tract metabolism. This contrasts with the pig where, following a more severe endotoxin challenge supplied enterally, increases in net portal vein appearance for several AA were approximately double that supplied from the diet, suggestive of mobilisation of intestinal tissue[4].

In the current study, glutamine showed lowered net removal in response to LPS. This has similarities to observations in both pigs post-surgery[35] and tumour-bearing rats[17] where the gut consumed less glutamine, possibly in response to lowered arterial concentrations. Indeed, arterial glutamine, and thus systemic supply, was reduced by 38% in the current study

Under control conditions, hepatic AA removal followed patterns previously reported[36,37], with most of the net absorbed histidine and phenylalanine extracted, but with only limited uptake of the branch-chain AA (17-27%). There was net output of glutamate but, contrary to earlier observations, this was not balanced by similar net hepatic glutamine removal[23,38]. More N was extracted from plasma by the liver as combined AA-N and ammonia-N than was released as urea-N, compatible with hepatic needs to support other processes[23], including synthesis of constitutive and export proteins. Part of the difference (4.5 mmol/h) would be used to support the measured albumin synthesis (1.4 mmol-N/h), but would be insufficient to account for estimated total plasma protein synthesis (7.2 mmol-N/h), although not all plasma proteins are synthesised by the liver (e.g. globulins).

LPS infusion increased hepatic removal for several AA, including glutamine, leucine, lysine, proline and threonine, and these contributed to the additional 6.5 mmol AA-N/h net removal by the liver, similar to the 8.3 mmol-N/h estimated from the change in total liver ILR (from Table 7). How much of this additional uptake of AA-N is catabolised is unclear because the numerical change in arterial urea was not supported by increased hepatic ureagenesis. Studies in humans have reported increased AA catabolism due to LPS, including hepatic oxidation of leucine[27], greater fractional extraction of leucine across the splanchnic bed during sepsis[39] and elevated whole body conversion of phenylalanine to tyrosine[40], primarily a liver event. In addition, infection or inflammation stimulate synthesis of constitutive and/or export proteins[1,4,33,41].

Phenylalanine is unusual in that the arterial concentration increases during endotoxaemia in pigs[4] and sheep[3], although not humans[27,28]. Hepatic removal of phenylalanine remained unchanged by LPS treatment even though it has been suggested that liver demands for phenylalanine (and tryptophan) would increase during infection and inflammation due to their relatively high abundance in positive acute phase proteins[7], the synthesis of which is increased by infection[42]. In the current study, such demands would be offset by simultaneous decreased synthesis (-50%) of albumin, a negative acute phase protein that contains 6% (w/w) phenylalanine. Therefore, the increased arterial plasma phenylalanine probably relates to mobilisation of protein from non-splanchnic tissues, particularly skeletal muscle[1,43].

Effect of AA supplementation

Targeted supply of nutrients in response to specific physiological, developmental or environmental events is a key nutritional aim. In various clinical situations much attention has been focused on demands for specific AA. For example, supplementation with large amounts of glutamine has been proposed for a variety of surgical and clinical states[16,44-46] associated with specific needs for the immune system[47]. In addition, claims as effective therapies have been made for a number of other AA supplied alone[48-50]. Other benefits have involved AA in combination[13,14], although these have not always been successful[51].

A recent approach involved dynamic measurements in sheep subjected to an LPS challenge in order to quantify the demands for specific AA[3], and these findings were applied within the current protocol. The success of such an approach can be assessed at several levels. The simplest involves the effect of supplementation on plasma AA concentrations and this produced statistical restoration to the saline-infused values for 5 of the AA, although further numerical improvement would be preferred for cysteine and serine. In contrast, threonine was probably over-supplied based on previous sheep data[3]and there may be differences between studies in the sensitivity of the animals to the LPS dose. Notably, earlier LPS caused an 80% reduction in plasma threonine[3], while for the current animals the decrease was only 56% and so less than actually given might have been needed to restore to control values. Recovery of AA infused into the post-absorptive venous drainage was not different from unity, except in the case of cysteine, possibly due to the requirement for synthesis of taurine and glutathione, processes that occur within the intestinal cells[52,53].

Based on the hypothesis that AA are mobilised from tissue protein, particularly muscle[43,54], to combat inflammation and if one (or more) of these AA are needed in considerable amounts then this will leave the remainder that are released in excess to requirements for protein synthesis. These will then be removed from the body via ureagenesis and lead to depletion of plasma concentrations. If the supplement contains those AA needed to support the anti-inflammatory responses, then this should reduce the need for peripheral tissue mobilisation and lower the overall hepatic removal for catabolic purposes. Despite the reasonable success in restoring the plasma concentrations of the supplemented AA to normal, there was no improvement in the plasma concentrations of the non-supplemented AA and neither was there a reduction in their removal by the liver between the LPS and LPS+AA treatments. For the supplemented AA, only for glutamine was there complete removal of the extra provided. This suggests that the increased arterial glutamine concentrations are probably due to mobilisation from other tissues; muscle is an obvious candidate where milli-molar quantities of free glutamine are present[55] and which is released during severe illness, even in the presence of supplemental glutamine[56]. For the other supplemented AA, hepatic removal ranged from 55% (proline) to 84% (serine) so the fates were partitioned between increased post-hepatic delivery, necessary to restore arterial concentrations to, or above, normal and potential support of liver-based mechanisms. The latter did not, however, involve restoration of the synthesis of the negative acute phase protein, albumin. This non-response was despite the fact that the increased hepatic uptake of the supplemented AA would, in theory, have the potential to support 30-130 g/d of albumin synthesis, in considerable excess of the 1g/d decrease observed. Similarly, the additional AA supplement did not alter rates of synthesis of lymphocytes. These observations might suggest that either other AA are needed in the supplement or that the immediate events during inflammation are less responsive to supplementation, e.g. there is a metabolic 'over-ride', and perhaps the focus should be on longer–term responses and enhanced recovery, as has been shown to occur in rats treated with exotoxin and supplemented with AA[14].

In summary, infusion of 6 AA predicted as key requirements during the response to an LPS challenge restored or exceed arterial concentrations of these to control values but not any of the other non-infused AA. Similarly, there was no restoration of the synthesis of the negative acute-phase protein, albumin, and no change in the elevated protein synthesis of lymphocytes. The parenteral infusion of the low dose of LPS did not affect gut metabolism or net AA absorption except for glutamine, where net removal was reduced. Hepatic uptake of leucine, lysine, glutamine and serine was increased by LPS, with liver removal of the latter two plus the other 4 infused AA increased during the AA supplementation. The data suggest that AA supplementation does not mitigate certain metabolic demands during the acute phase of an endotoxin challenge but whether supplementation provides benefits on later responses and the period of recovery requires further investigation.

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Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authorship

The contributions of the authors were as follows: C.J.McN., S.O.H., G.H. and G. E. L. were responsible for study concept and design. C.J.McN, S.O.H., G. E. L. and D. M. B. were responsible for data collection and collation. G. E. L. and G. H. were responsible for data analysis and statistical matters. C.J.McN., S.O.H., G. E. L. and G. H. were responsible for the first draft and critical revision of the manuscript for important intellectual content.

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Table 1. Impact of a 20 h infusion of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA), on arterial concentrations of albumin, total protein, glucose, lactate and lymphocytes in 8 sheep.

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS+AA	SED	Treatment*	Period*	LPS status†
Albumin (g/l)	27.3[a]	26.7[ab]	25.8[b]	0.48	0.021	0.022	0.007
Protein (g/l)	62.2	59.9	58.1	1.85	0.059	NS	0.018
Glucose (mM)	4.09	2.59	3.25	0.567	0.070	NS	0.067
Lactate (mM)	1.42	1.19	1.56	0.566	NS	NS	NS
Lymphocytes (10^9 cells/l) ‡	4.58[a]	11.04[b]	10.74[b]	1.182	0.002	NS	<0.001

LPS lipopolysaccharide, AA amino acids infused

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. There were no period x treatment effects ($P>0.05$). Where there was a treatment effect ($P<0.05$), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different ($P<0.05$).

† Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

‡ One missing value (for LPS treatment)

Table 2. Effect of 20 h infusion of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA), on synthesis rates of plasma albumin, total plasma protein and lymphocytes in 8 sheep

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS+AA	SED	Treatment*	Period*	LPS status†
Albumin							
FSR (%/d)	4.88[a]	2.22[b]	2.52[b]	0.462	<0.001	NS	<0.001
ASR (g/d)	2.92[a]	1.35[b]	1.38[b]	0.430	0.004	NS	0.001
Total protein							
FSR (%/d)	11.4[a]	16.2[b]	15.1[b]	1.49	0.043	NS	0.012
ASR (g/d) ‡	15.2[a]	19.2[ab]	21.2[b]	1.83	0.048	NS	0.007
Lymphocytes							
FSR (%/d)	6.33[a]	10.95[b]	9.68[ab]	1.75	0.025	0.032§	0.043§
ASR (mg/d)**	25.0[a]	91.2[b]	101.5[b]	19.85	<0.001	0.034§	<0.001

LPS lipopolysaccharide, AA amino acids infused, FSR fractional synthesis rate, ASR absolute synthesis rate

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

[†] Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

[‡] Period x treatment effect (P=0.029) values lower during period 2 for LPS treatment (similar between periods for saline and LPS+AA infusions).

[§] Values lower during period 2.

[**] One missing value (LPS)

Table 3. Effect of a 20 h infusion of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA), on plasma arterial concentrations (µmol/kg) of amino acids in 8 sheep.

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS+AA	SED	Treatment*	Period*	LPS†
Non-infused AA							
Alanine	162[a]	105[b]	98[b]	12.7	0.001	NS	<0.001
Arginine	164[a]	79[b]	106[b]	20.6	0.005	0.085‡	<0.001
Aspartate	8[a]	4[b]	5[b]	1.27	0.015	NS	0.009
Glutamate	83[a]	53[b]	58[b]	9.5	0.015	NS	0.005
Glycine	477[a]	255[b]	217[b]	36.5	<0.001	NS	<0.001
Histidine	60[a]	44[b]	50[ab]	5.1	0.026	NS	0.009
Isoleucine	74[a]	49[b]	42[b]	7.2	<0.001	NS	<0.001
Leucine	78[a]	69[ab]	54[b]	7.1	0.015	NS	0.025

Lysine	124[a]	66[b]	55[b]	14.7	0.001	0.042[‡]	<0.001
Phenylalanine	45[a]	60[b]	58[b]	2.2	<0.001	NS[§]	<0.001
Tryptophan	31[a]	25[b]	28[ab]	2.2	0.100	NS	0.079
Tyrosine	59[a]	44[b]	45[ab]	7.2	0.041	NS	0.007
Valine	163[a]	132[b]	110[b]	14.2	0.006	NS	0.002
Urea	4199[a]	5105[a]	5151[a]	583.0	0.086	NS	0.014
Infused AA							
Cysteine	86[a]	60[b]	82[ab]	9.1	0.035	NS	0.083
Glutamine	333[a]	207[b]	301[a]	20.6	<0.001	0.041[‡¶]	0.038
Methionine	22[a]	7[b]	28[a]	5.0	0.015	0.034[‡]	NS
Proline	73[a]	40[b]	77[a]	11.8	0.030	NS	NS
Serine	56[a]	23[b]	44[a]	7.4	0.008	NS	0.037
Threonine	86[a]	38[a]	144[b]	24.0	0.007	NS[¶]	NS

LPS lipopolysaccharide, AA amino acids infused

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

[†] Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

[‡] Values greater in Period 2.

[§] Period x Treatment effect (P=0.009), with lower values for LPS+AA during Period 2.

[¶] Period x Treatment effect (P=0.003 for glutamine,; P=0.04 for threonine), with greater values for LPS+AA during Period 2.

Table 4. Net PDV supply (absorbed from diet + any infused AA) of amino acid-N (µmol N/min) in 8 sheep in response to 20 h infusions of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA).

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS+AA	SED[+]	Treatment[*]	Period[*]	LPS[†]
Non-infused AA-N							
Alanine	31.3[ab]	24.0	39.9[c]	3.06	0.015	NS	NS
Arginine	34.2[a]	19.6[b]	60.2[c]	4.32	0.006	0.017[‡]	NS
Aspartate	4.3	3.4	3.5	1.50	NS[¶]	NS	NS
Glutamate	5.1	14.8	13.0	5.03	NS	NS	0.083
Glycine	20.0	18.1	25.2	2.68	NS	NS	NS
Histidine	18.6	15.0	19.0	2.58	NS	NS	NS
Isoleucine	15.5	12.3	17.3	2.28	NS	0.053[‡]	NS
Leucine	19.1	15.0	22.9	2.60	0.079	NS	NS

Lysine[§]	40.6[a]	30.3[b]	51.3[c]	4.43	0.021	0.034[‡]	NS
Phenylalanine	12.3	9.7	13.8	1.36	0.075	0.062[‡]	NS
Tryptophan	3.7	2.5	6.0	1.57	NS	0.093[‡]	NS
Tyrosine	10.5	7.9	12.2	1.41	0.084	0.069[‡]	NS
Valine	17.4	13.4	19.3	2.58	NS	NS	NS
Urea-N	-259	-359	-329	101.5	NS	NS	NS
Ammonia	547	433	807	139.3	0.083	NS	NS
Infused AA-N[§]							
Cysteine	1.9[a]	1.8[a]	15.5[b]	0.79	<0.001	NS	0.052
Glutamine	-51.6[a]	-17.6[b]	27.4[c]	11.59	<0.001	NS	0.002
Methionine	6.4[a]	6.2[a]	17.7[b]	1.09	<0.001	NS	0.071
Proline	10.9[a]	10.2[a]	26.0[b]	1.95	<0.001	NS	NS
Serine	24.1[a]	21.0[a]	55.0[b]	4.62	<0.001	NS	NS
Threonine	13.8[a]	12.2[a]	46.7[b]	2.73	<0.001	NS	0.099

PDV portal-drained viscera, LPS lipopolysaccharide, AA amino acids infused

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. There were no period x treatment effects (P>0.05). Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

† Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

‡ Values lower for Period 1 than Period 2 except for arginine where Period 1 was greater.

§ infusion rates (µmol-N/min) into mesenteric vein, 17.8 (proline), 10.8 (methionine), 34.7 (serine), 38.4 (threonine), 19.2 (cysteine), 89.2 (glutamine) for LPS+AA treatment.

¶ Only 3 sheep for the LPS+AA treatment

Table 5. Net hepatic removals (µmol N/min) of amino acid-N in 8 sheep in response to 20 h infusions of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA).

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS + AA	SED[+]	Treatment[*]	Period[*]	LPS[†]
Non-infused AA-N							
Alanine	-24.8[a]	-35.6[b]	-36.7[b]	5.05	0.028	0.052	0.004
Arginine	-25.9[a]	-42.9[ab]	-60.6[b]	9.51	0.032[‡]	NS	0.031
Aspartate	-0.6	-2.0	-1.2	1.08	NS	NS[§]	NS
Glutamate	16.8	6.4	-4.7	6.70	NS	NS	NS
Glycine	-30.5	-38.1	-35.8	5.18	NS	NS	NS
Histidine	-18.9	-21.8	-20.8	2.06	NS	0.020[‖]	0.058
Isoleucine	-2.6	-4.9	-5.3	0.96	0.074	NS[§]	0.036
Leucine	-5.1[a]	-8.0[b]	-8.0[b]	0.96	0.009	0.020[§‖]	0.008

Lysine	-12.9[a]	-18.7[b]	-24.3[b]	2.61	0.004	0.099[§]	0.009
Phenylalanine	-10.9	-12.5	-13.3	1.39	NS	NS	0.029
Tryptophan	-3.5	-3.6	-8.3	1.93	0.052	NS	NS
Tyrosine	-9.6	-9.6	-9.4	1.19	NS	NS[§]	NS
Valine	-4.0	-5.6	0.2	3.44	NS	NS	NS
Urea	658	614	802	161.1	NS	NS	NS
Ammonia	-579	-456	-824	138.7	0.088	NS	NS
Infused AA-N							
Cysteine	-0.8[a]	-1.8[a]	-8.7[b]	0.70	<0.001	NS	0.017
Glutamine	3.8[a]	-38.5[b]	-78.2[c]	18.6	0.004	NS	0.003
Methionine	-3.6[a]	-4.4[a]	-11.5[b]	1.73	<0.001	NS	0.067
Proline	-5.8[a]	-8.8[b]	-14.2[c]	1.05	<0.001	0.014[§]	0.005
Serine	-12.7[a]	-15.3[a]	-37.1[b]	3.12	<0.001	NS	0.034
Threonine	-5.5[a]	-6.9[b]	-32.0[c]	0.90	<0.001	NS	0.036

Total AA-N	-154[a]	-271[b]	-382[c]	38.4	<0.001	NS	0.001

LPS lipopolysaccharide, AA amino acids infused

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

† Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

‡ Only 3 sheep for the LPS+AA treatment

§ Period x treatment interaction (P<0.05) with less uptake during Period 2 for LPS and LPS+AA.

‖ More hepatic removal during Period 1

Table 6. Net total splanchnic appearances (µmol N/min) of amino acid-N in response to 20 h infusion of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA),LPS, in 8 sheep.

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS+AA	SED[+]	Treatment[*]	Period[*]	LPS[†]
Non-infused AA-N							
Alanine	6.5[a]	-11.9[b]	3.6[a]	3.93	0.006	0.005[‡]	0.030
Arginine	8.4[a]	-20.4[b]	-2.9[ab]	10.01	0.048[¶]	NS	0.043
Aspartate	3.7	1.5	2.2	1.09	0.084	0.060	0.013
Glutamate	21.9	20.4	18.5	9.09	NS	NS	NS
Glycine	-10.6	-15.8	-14.7	5.65	NS	NS	NS
Histidine	-0.3	-3.1	-5.4	2.85	NS	0.070	0.083
Isoleucine	12.9	8.1	11.3	3.17	NS	0.060	0.099
Leucine	14.0	7.5	14.3	2.64	0.054	0.026[‡]	NS

Lysine	27.8	14.0	24.7	5.90	0.077	0.044[‡]	0.097
Phenylalanine	1.5	-2.1	-0.2	1.34	0.059	0.059	0.071
Tryptophan	0.1[a]	-1.1[ab]	-2.3[b]	0.67	0.010	NS	0.024
Tyrosine	0.9	-1.2	2.3	1.22	0.073	0.022[‡]	NS
Valine	13.5	8.0	19.3	4.27	0.096	0.066	NS
Urea-N	399	288	440	144.7	NS	NS	NS
Ammonia	-32	-22	18	14.7	NS	NS[§]	NS
Infused AA-N							
Cysteine	1.1[a]	0.2[a]	6.6[b]	1.05	0.001	NS	NS
Glutamine	-47.9	-53.0	-53.9	25.55	NS	NS	NS
Methionine	2.8[a]	2.1[a]	5.8[b]	0.082	0.006	0.070[§]	NS
Proline	5.1[a]	1.2[a]	11.9[b]	1.89	0.004	0.026[‡]	NS
Serine	11.4	6.0	17.0	3.94	0.081	0.088	NS
Threonine	8.3[a]	4.8[a]	15.2[b]	2.57	0.004	NS	NS

Net AA-N	79[a]	-22[b]	63[ab]	36.9	0.069	0.028‡	NS

TSP total splanchnic release, LPS lipopolysaccharide, AA amino acids infused

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

† Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

‡ Values lower for Period 1 than Period.

§ Period x treatment interaction for methionine with lower TSP (P=0.014) during Period 2 for saline infusion while values for LPS and LPS+AA infusions were greater.

¶ Only 3 sheep for the LPS+AA treatment

Table 7. Impact of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA), on irreversible loss rates (mmol/h) of plasma phenylalanine for the whole body (WB) and across the tissues of the splanchnic bed.

(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment*				*P*		
	Saline	LPS	LPS+AA	SED[+]	Treatment[†]	Period[†]	LPS[‡]
Whole Body	2.28[a]	2.58[b]	2.74[b]	0.097	0.003	0.096[§]	<0.001
Splanchnic tissues							
PDV	-0.31	-0.35	-0.34	0.101	NS	0.047[¶]	NS
Liver	-0.57[a]	-0.72[ab]	-0.83[b]	0.111	0.046	NS	0.023
TSP	-0.87	-1.09	-1.15	0.167	NS	NS	0.034
Tissue:WB ratio							
PDV	0.13	0.13	0.13	0.029	NS	0.072[¶]	NS
Liver	0.25	0.28	0.30	0.053	NS	NS	NS
TSP	0.39	0.43	0.42	0.076	NS	NS	NS

WB ILR whole body irreversible loss rate (of phenylalanine), TSP total splanchnic preparation (liver + PDV); PDV, portal drained viscera (total gut)

* all values based on arterial enrichments

† Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS, or LPS+AA infusion. There were no period x treatment effects ($P>0.05$). Where there was a treatment effect ($P<0.05$), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different ($P<0.05$).

‡ Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

§ Values greater for Period 1 than Period 2.

¶ Values greater for Period 2 than Period 1.

SUPPLEMENTARY DATA

Supplementary Table S1

Impact of a 20 h infusion of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA), on arterial concentrations of albumin, total protein, glucose, lactate and lymphocytes in 11 sheep.
(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				P		
	Control	LPS	LPSAA	SED	Treatment*	Period*	LPS[†]
Albumin (g/l)	27.3	26.3	25.9	1.10	NS	NS	NS
Protein (g/l)	61.9	60.9	59.3	2.35	NS	NS	NS
Glucose (mM)[¶]	4.12	2.78	3.27	0.487	0.019	NS	0.006
Lactate (mM)	1.34	1.13	1.38	0.428	NS	NS	NS
Lymphocytes (10^9 cells/l)[‡]	4.33[a]	12.82[b]	9.67[b]	1.917	<0.001	NS	<0.001

* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS (n=6), or LPS+AA (n=5) infusion. There were no period x treatment effects (P>0.05). Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

[†] Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

[‡] One missing value (for LPS treatment)

Supplementary Table S2

Effect of 20 h infusion of saline or lipopolysaccharide (LPS; 2 ng/kg LW per min), either with or without six supplemental amino acids (AA), on synthesis rates of plasma albumin, total plasma protein and lymphocytes in 11 sheep
(Predicted means with the standard errors of the difference (SED) between means for the effect of treatment)

	treatment				*P*		
	Saline	LPS	LPS+AA	SED	Treatment*	Period*	LPS status†
Albumin							
FSR (%/d)	4.67[a]	2.00[b]	2.77[b]	0.634	<0.001	NS	<0.001
ASR (g/d)	2.92[a]	1.35[b]	1.38[b]	0.412	<0.001	NS	<0.001
Total protein							
FSR (%/d)	11.4[a]	16.2[b]	15.1[b]	1.31	<0.001	0.020‡	<0.001‡
ASR (g/d)	15.2[a]	19.2[b]	21.2[b]	1.72	0.006	0.012‡	0.002‡
Lymphocytes							
FSR (%/d)	6.33[a]	10.95[b]	9.68[ab]	2.25	0.042	0.017‡	0.027‡
ASR (mg/d) §	26.3[a]	112.9[b]	90.5[b]	31.63	0.006	0.036‡	0.002‡

LPS lipopolysaccharide, AA amino acids infused, FSR fractional synthesis rate, ASR absolute synthesis rate
* Analysed by random effects model, with sheep and period within sheep as random effects and period, treatment plus their interaction as fixed effects, where treatment was either saline, LPS (n=6), or LPS+AA (n=5) infusion. There were no period x treatment effects (P>0.05). Where there was a treatment effect (P<0.05), post-hoc t-test was performed to compare the treatment means, where values in rows with unlike superscripts are significantly different (P<0.05).

† Analysed by random effects model as described above, where treatment reflects LPS status, i.e. either saline or LPS (both alone and in combination with AA).

‡ Values lower during period 2.

§ One missing value (LPS).