Plasma and muscle connective tissue protein analyses

Production of intrinsically labeled protein

Intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine-labeled casein protein was obtained during the constant infusion of L-[1-^{13}C]-phenylalanine (455 μmol·kg^{-1}·min^{-1}) and L-[1-^{13}C]-leucine (200 μmol·kg^{-1}·min^{-1}) maintained for 96 h in a lactating dairy cow. The milk was collected, processed, and fractionated into the casein protein concentrate as previously described. The L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments in casein protein were measured by gas chromatography-combustion–isotope ratio mass spectrometry (GC-IRMS; MAT 252, Finnigan, Breman, Germany) and averaged 38.7 molar percent excess (MPE) and 9.3 MPE, respectively. The protein met all chemical and bacteriological specifications for human consumption.

Tracer preparation and infusion

The stable isotope tracers L-[*ring*-^{2}H$_5$]-phenylalanine, L-[1-^{13}C]-leucine, and L-[*ring*-^{2}H$_2$]-tyrosine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9% saline before infusion (Basic Pharma, Geleen, The Netherlands). Continuous intravenous infusions were performed using a calibrated IVAC 598 pump (San Diego, CA).

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (GLUC3, Roche, Ref: 05168791 190, and Immunologic, Roche, Ref: 12017547 122, respectively). Quantification of plasma amino acid concentrations was performed using ultra-performance liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). 50 µl of blood plasma was deproteinized using 100 µL of 10 % SSA with 50 µM of MSK-A2 internal standard (Cambridge Isotope Laboratories,

Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged (15 min at 14000 RPM). After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 µL of AccQ-Tag derivatizing reagent solution (Waters, Saint-Quentin, France) was added after which the solution was heated to 55 °C for 10 min. An aliquot of 1 µL was injected and measured using UPLC-MS. Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Wilmington, Delaware, USA). The plasma phenylalanine and leucine ^{13}C and ^{2}H enrichments were determined using selective ion monitoring at m/z 302 and 303 for unlabeled and labeled (1-^{13}C) leucine, respectively; m/z 336, 337, and 341 for unlabeled and labeled (1-^{13}C and *ring*-$^{2}H_5$) phenylalanine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation.

Muscle protein-bound amino acid enrichment analysis

Following hydrolyzation, the free amino acids were then dissolved in 25 % acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA), washed 5 times with water and finally eluted with 2 M NH_4OH. To determine connective protein L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments by GC-IRMS analysis, the purified amino acids were converted into N-ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate (ECF). The L-[*ring*-$^{2}H_5$]-phenylalanine derivatives were measured using a gas chromatography-isotope ratio mass spectrometer (MAT 253; Thermo Fisher Scientific, Bremen, Germany) equipped with a pyrolysis oven (GC-P-IRMS) and a 60 m DB-17MS column and 5 m precolumn (No. 122–4762; Agilent) and GC-Isolink. Ion masses 1 and 2 were

monitored to determine the $^{2}H/^{1}H$ ratios of muscle protein bound phenylalanine. The derivatized L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine samples were measured using a gas chromatography-isotope ratio mass spectrometer (Finnigan MAT 252; Thermo Fisher Scientific, Bremen, Germany) equipped with a Ultra I GC-column (no. 19091A-112; Hewlett-Packard, Palo Alto, CA) and combustion interface II (GC-C-IRMS). Ion masses 44, 45, and 46 were monitored for $^{13}C/^{12}C$ phenylalanine and leucine, respectively. By establishing the relationship between the enrichment of a series of L-[1-^{13}C]phenylalanine, L-[1-^{13}C]leucine, and L-[*ring*-$^{2}H_5$]-phenylalanine standards of variable enrichment and the enrichment of the *N(O,S)*-ethoxycarbonyl ethyl esters of these standards, the muscle-protein-bound enrichment of phenylalanine and leucine was determined.