The production of intrinsically labeled milk and meat protein is feasible and provides functional tools for human nutrition research

B. Pennings,*† W. F. Pellikaan,‡ J. M. G. Senden,*† A. M. van Vuuren,§ J. Sikkema,* and L. J. C. van Loon*†¹

*Top Institute Food and Nutrition (TIFN), 6700 AN Wageningen, the Netherlands

†Department of Human Movement Sciences, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Centre+ (MUMC+), 6229 ER Maastricht, the Netherlands

‡Animal Nutrition Group, Wageningen University, 6700 AH Wageningen, the Netherlands

§Wageningen UR Livestock Research, 8200 AB Lelystad, the Netherlands

ABSTRACT

Administration of labeled, free amino acids does not allow direct assessment of in vivo dietary protein digestion and absorption kinetics. Consequently, dietary protein sources with labeled amino acids incorporated within their protein matrix are required. The aim of the present study was to produce intrinsically L-[1-¹³C]phenylalanine-labeled milk and meat protein that would permit in vivo assessment of postprandial protein digestion and absorption kinetics in humans. One lactating dairy cow was continuously infused with 420 μ mol of L-[1-¹³C]phenylalanine/min for 96 h, with plasma and milk being collected before, during, and after isotope infusion. Twenty-four hours after infusion, the cow was slaughtered to produce intrinsically labeled meat. Levels of L-[1-¹³C]phenylalanine enrichment as high as 40 mole percent excess (MPE) in milk and 1.5 MPE in meat protein were achieved. In a subsequent human proof-of-principle experiment, 2 healthy young males (25 ± 1 yr; 66.2 ± 5.2 kg) each ingested 135 g of L-[1-¹³C]phenylalanine intrinsically labeled minced beef, after which plasma samples were collected at regular time intervals. Plasma L-[1-¹³C]phenylalanine enrichments increased during the first 90 min following beef ingestion, reaching peak plasma enrichment levels of 0.61 ± 0.04 MPE. Whole-body net protein balance, assessed by continuous infusion of L-[ring-²H₅]phenylalanine and L-[ring-²H₂]tyrosine, was higher in the postprandial period compared with basal values (6.4 ± 0.1 vs. -4.5 ± 0.1 μ mol/kg per h). In conclusion, the production of intrinsically L-[1-¹³C]phenylalanine-labeled milk and meat protein is feasible and provides functional tools to investigate in vivo protein digestion and absorption kinetics in humans.

Key words: digestion, absorption, dietary protein, stable isotope

INTRODUCTION

For many years, stable isotope-labeled amino acids have been applied to study protein metabolism in vivo in humans (Wagenmakers, 1999). This work has led to important physiological insights and has provided a wealth of practical information on the effect of nutrition in maintaining or increasing lean tissue mass in both health and disease (Koopman et al., 2007). In general, the turnover of a specific amino acid is investigated through isotope dilution via continuous intravenous administration of a labeled amino acid. However, when investigating the effect of feeding on protein metabolism, most groups generally apply a research design in which labeled leucine or phenylalanine is co-ingested, with or without continuous infusion of an intravenous amino acid tracer. The fact that the oral tracer is being administered as a free amino acid and the bulk of amino acids are provided as intact dietary protein introduces substantial methodological limitations (Boirie et al., 1996). Free amino acids and protein-derived amino acids exhibit an entirely different timing and efficiency of intestinal absorption (Dangin et al., 2002). Therefore, dietary protein with a labeled amino acid incorporated within its protein matrix is warranted to directly assess the digestion and absorption kinetics of dietary protein in vivo in humans. However, such intrinsically labeled dietary protein is not commercially available and only a few reports describe the production of stable isotope-labeled proteins for more specific purposes (Berthold et al., 1991; Boirie et al., 1995; Evenepoel et al., 1997; Deglaire et al., 2008).

Previously, intrinsically labeled milk protein has been produced by intravenous infusion of L-[1-¹³C]leucine (Boirie et al., 1995) or L-[1-¹³C]phenylalanine (Van Loon et al., 2009) in lactating dairy cows. In the latter experiment, L-[1-¹³C]phenylalanine enrichments in the collected milk protein as high as 29.4 mole percent excess (MPE) were achieved. Such high enrichment levels are necessary when aiming to assess both in vivo protein digestion and absorption kinetics as well as the

Received April 14, 2011.

Accepted May 18, 2011.

¹Corresponding author: L.vanLoon@maastrichtuniversity.nl

subsequent postprandial muscle protein synthetic response following ingestion of meal-like amounts (20–35 g) of protein (Koopman et al., 2009a; Pennings et al., 2011). As the latter becomes challenging with smaller amounts of dietary protein, we aimed to produce milk proteins with an even greater enrichment level. To allow maintenance of high plasma L-[1-¹³C]phenylalanine levels, a single Holstein-Friesian dairy cow was continuously infused with 400 g of L-[1-¹³C]phenylalanine for up to 4 d (96 h). Furthermore, an isotonic glucose solution was infused simultaneously to increase the mean extraction rate of plasma L-[1-¹³C]phenylalanine by the mammary gland (Schei et al., 2007) and improve milk protein production by minimizing the loss of tracer due to hepatic amino acid oxidation (Vanhatalo et al., 2003).

The maximal recovery rate of tracer in milk protein has been estimated to be 25 to 28% (Boirie et al., 1995; Van Loon et al., 2009). We hypothesized that the current infusion protocol would also allow a large amount of L-[1-¹³C]phenylalanine to be incorporated in the skeletal muscle tissue of the animal. As a consequence, we speculated whether the L-[1-¹³C]phenylalanine enrichment of meat protein would be detectable and potentially even allow subsequent application in human nutrition research. Therefore, the cow was slaughtered 24 h after cessation of the stable isotope infusion procedure and the meat was subsequently used in a human proof-of-principle study to determine the potential of assessing beef protein digestion and absorption kinetics in vivo in humans.

In the present study, we extend on our previous work (Van Loon et al., 2009) by investigating the potential to produce intrinsically L-[1-¹³C]phenylalanine-labeled milk protein with an enrichment level that is greater than any previously produced batch of labeled milk protein. Furthermore, we tested the applicability of this approach to produce intrinsically L-[1-¹³C]phenylalanine-labeled meat protein that would allow the in vivo assessment of protein digestion and absorption kinetics following beef ingestion in humans.

MATERIALS AND METHODS

Production of Intrinsically Labeled Protein

Animal. One lactating Holstein-Friesian dairy cow (fifth parity, 642 kg of live weight, 40 DIM at the start of the infusion period) was selected for the production of intrinsically L-[1-¹³C]phenylalanine-labeled milk and meat protein. During the infusion period, the cow produced 48.6 ± 0.6 kg of milk/d, with an equivalent amount of 2.05 ± 0.002 kg of fat and 1.38 ± 0.002 kg of protein per day. The animal was fed a mixed ration

of grass silage (50.8% wt/wt), maize silage (33.9% wt/wt), corn cob (11.3% wt/wt), soy-rape seed mix (3.4% wt/wt), lime (0.1% wt/wt), salt (0.1% wt/wt), and a mineral mix (0.3% wt/wt). During the experiment, the cow consumed 23.5 ± 1.0 kg of DM per day, which covered 82% of NE_L requirements and 80% of the protein requirements (Van Es, 1978; Tamminga et al., 1994). The forage mixture was offered ad libitum 3 times daily allowing 10%orts. Equal portions of concentrate (7.1 kg of DM/d) were given at 0600, 1400, and 2200 h. The animal was housed in an individual tie stall and had free access to water. The experiment and animal handling procedures were approved by the Ethics Committee of the Wageningen University under the Dutch Law on Animal Experimentation.

Stable Isotope Infusion. The stable isotope L-[1-¹³C]phenylalanine (99% enriched) was purchased from Cambridge Isotopes Laboratories (Andover, MA). A total of 400 g of L-[1-¹³C]phenylalanine tracer was dissolved in 40 L of an isotonic glucose (5% wt/vol) solution (Braun Melsungen AG, Melsungen, Germany) with a final concentration of 278 mmol of glucose/L and 60.5 mmol of L-[1-¹³C]phenylalanine/L. Infusates were stored at 4°C pending further use. Prior to use, the infusion bags were allowed to warm up to room temperature.

An outline of the applied infusion protocol is shown in Figure 1. Five days before the start of the tracer infusion (d –5) the cow was adapted to 3 milkings per d (0600, 1400, and 2200 h). Two days before tracer infusion (d –2), catheters (Careflow 16 gauge $\times$ 300 mm with 18 gauge $\times$ 70-mm needle introducer; Becton Dickinson, Breda, the Netherlands) were inserted percutaneously in the right and left jugular veins for intravenous infusion and blood sampling, respectively, as described previously (Van Loon et al., 2009). Directly following catheterization, glucose was infused at a rate of 116 mmol/h. After 2 d, the milk from 2200 h (d –1) and 0600 h (d 0) was collected (unlabeled milk) and the continuous infusion of glucose + L-[1-¹³C]phenylalanine was started directly following the 0600 h milking on d 0. This combined glucose and tracer infusion was continued for 96 h until 0600 h on d 4, with L-[1-¹³C]phenylalanine infused at a rate of 6.94 mL/min (420 μ mol/min). General health of the cow was monitored continuously and all procedures were well tolerated.

Milk Collection. The cow was milked every 8 h until 24 h following cessation of the infusion (d 5), with blood samples being obtained at each milking. Milk obtained during each milking was separately collected in 25-L containers, which were directly cooled to <7°C, frozen within 24 h, and stored at –40°C within 48 h until further processing. The processing of the milk was performed at NIZO Food Research (Ede, the Nether-

Figure 1. Outline of the cow infusion protocol.

lands) as described previously (van Loon et al., 2009). In short, intrinsically L-[1-¹³C]phenylalanine-labeled milk was fractionated into a casein and a whey protein concentrate. Before the fractionation procedure, collected milk was thawed and pooled into low- and high-labeled batches. The high-labeled batch consisted of milk collected from 2200 h on d 0 to 0600 h on d 4, from 16 to 96 h following the onset of the tracer infusion, respectively. The low-labeled batch consisted of milk collected from 2200 h at d -1 to 0600 h on d 0 (unlabeled milk), milk collected at 1400 h on d 0 (8 h following the start of the tracer infusion), and milk collected from 1400 h on d 4 to 0600 h on d 5 (24 h following cessation of the tracer infusion).

Meat Collection. Twenty-four hours after the infusion period, the cow was transported to a commercial abattoir (Henk Worst, Nijkerk, the Netherlands) and slaughtered. The carcass was refrigerated at 4°C for 2 d postmortem. Thereafter, the carcass was deboned and meat cuts from the loin rib, shoulder, and leg were weighted in portions of 135 g and stored at -18°C. Before packaging, part of the lean beef was minced. Slaughter and processing of the meat followed strict Food and Consumer Product Safety Authority regulations to ensure that no contamination occurred during processing of the meat and that meat products were fully qualified for human consumption.

Cow Plasma and Protein Analyses. Plasma phenylalanine and tyrosine were derivatized to their *tert*-butyldimethylsilyl (TBDMS) derivatives, and their ¹³C enrichments were determined by electron ionization GC-MS (Agilent 6890N GC/5973N MSD, Agilent, Wilmington, DE) using selected ion monitoring of masses 336 and 337 for unlabeled and labeled L-[1-¹³C]phenylalanine, respectively; and masses 466 and 467 for unlabeled and labeled L-[1-¹³C]tyrosine, respectively (Wolfe and Chinkes, 2005).

For measurement of L-[1-¹³C]phenylalanine and L-[1-¹³C]tyrosine enrichments in the intrinsically labeled milk protein, 100 μL of milk was added to 900 μL of 2% ice-cold perchloric acid (PCA). The mixture was vortexed, kept on ice for 10 min, and centrifuged.

The protein pellet was washed with 3 additional 1.5-mL washes of 2% PCA, dried, and hydrolyzed in 6 M

HCl at 120°C for 15 to 18 h. The hydrolyzed protein fraction was dried under a nitrogen stream while being heated to 120°C, after which 50% acetic acid solution was added. The hydrolyzed protein was passed over a Dowex exchange resin (AG 50W-X8, 100–200 mesh hydrogen form, BioRad, Hercules, CA) using 2 M NH₄OH. Thereafter, the eluate was dried and the purified amino acids were derivatized to their *N*(*O,S*)-ethoxycarbonyl ethyl esters for the determination of ¹³C/¹²C ratios of milk protein-bound phenylalanine and tyrosine (Husek, 1991). Thereafter, the derivative was measured by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS; Finnigan MAT 252, Bremen, Germany) using HP Ultra I GC-column (#19091A-112, Hewlett-Packard, Palo Alto, CA), combustion interface II, and monitoring of ion masses 44 and 45.

For measurement of L-[1-¹³C]phenylalanine enrichment in the intrinsically labeled meat protein, 55 mg of meat was freeze-dried. Collagen, blood, and other nonmuscle fiber material were removed from the meat under a light microscope. The isolated muscle fiber mass (10–15 mg) was weighed, and 8 volumes (8× dry weight of isolated muscle fibers × wet/dry ratio) of ice-cold 2% PCA were added. The tissue was then homogenized and centrifuged and the meat protein pellet was analyzed in a similar manner as the milk protein pellet.

The protein-bound enrichments of phenylalanine and tyrosine were determined by establishing the relationship between the enrichment of a series of L-[1-¹³C]phenylalanine and L-[1-¹³C]tyrosine standards of variable enrichment and the enrichment of the *N*(*O,S*)-ethoxycarbonyl ethyl esters of these standards. Standard regression curves were applied in all isotopic enrichment analyses to assess for linearity of the mass spectrometer and to control for the loss of tracer.

Human Proof-of-Principle Study

Subjects. Two healthy young male volunteers (age: 25 ± 1 yr; weight: 66.2 ± 5.2 kg; body mass index: 20.8 ± 1.6 kg/m²; basal glucose: 5.2 ± 0.3 mmol/L; basal insulin: 8.5 ± 1.8 mU/L) with no history of participating in any regular exercise program were selected to participate in the present study. Both subjects were

informed on the nature and possible risks of the experimental procedures before their written informed consent was obtained. This study was approved by the Medical Ethics Committee of the Academic Hospital Maastricht.

Protocol. At 0800 h, following an overnight fast, subjects arrived at the laboratory by car. A Teflon catheter was inserted into an antecubital vein for stable isotope infusion. A second Teflon catheter was inserted in a heated dorsal hand vein of the contralateral arm and placed in a hot-box (60°C) for arterialized blood sampling (Abumrad et al., 1981). Stable isotopes of L-[ring-²H₅]phenylalanine and L-[ring-²H₂]tyrosine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9% saline before infusion. Tracer solutions were prepared by the pharmacy of the Academic Hospital Maastricht (Maastricht, the Netherlands) following Good Manufacturing Practice standards. Following basal blood collection, the plasma phenylalanine and tyrosine pools were primed with a single intravenous dose of 2 µmol/kg L-[ring-²H₅]phenylalanine and 0.775 µmol/kg L-[ring-²H₂]tyrosine. Thereafter, continuous tracer infusion was started with an infusion rate of 0.050 and 0.019 µmol/kg per min for L-[ring-²H₅]phenylalanine and L-[ring-²H₂]tyrosine, respectively. After each subject had rested in a supine position for 60 min, a second arterialized blood sample was drawn ($t = -90$ min), marking the start of the post-absorptive period. During the post-absorptive period, arterialized blood samples were collected at $t = -60$, -30 , and 0 min. Subjects then received 135 g of minced beef (containing 35 g of protein). During the postprandial period, arterialized blood samples were collected at $t = 30$, 60, 90, 120, 150, 180, 240, 300, and 360 min.

Beef Preparation. Subjects received a portion of 135 g of minced lean beef. Meat was prepared in the kitchen of the Department of Human Movement Sciences (Maastricht University Medical Centre, Maastricht, the Netherlands) on the morning of the test day. In brief, the minced beef (thawed overnight in a refrigerator at 4°C) was grilled until the internal temperature reached 65°C. Subjects ingested the beef within 15 min. Subjects were allowed to add salt (0.5 g) to the beef and drink a glass of water (200 mL).

Plasma Analyses. Plasma (100 µL) for amino acid analyses was deproteinized on ice with 10 mg of dry 5-sulfosalicylic acid and mixed; the clear supernatant was collected after centrifugation at $1,000 \times g$ at 4°C for 10 min. Plasma amino acid concentrations were determined by HPLC after precolumn derivatization with *o*-phthalaldehyde (van Eijk et al., 1993). For plasma ring-²H enrichment measurements, phenylalanine and tyrosine were derivatized to their TBDMS derivatives, and their ²H enrichments were determined by electron

ionization GC-MS (Agilent 6890N GC/5973N MSD) using selected ion monitoring of masses 336 and 341 for unlabeled and labeled (ring-²H₅) phenylalanine, respectively, and masses 466, 468 and 470 for unlabeled and labeled (ring-²H₂ and ring-²H₄) tyrosine, respectively (Wolfe and Chinkes, 2005). For plasma 1-¹³C enrichment measurements, phenylalanine was derivatized to its *N*(*O,S*)-ethoxycarbonyl ethyl esters (Husek, 1991). Thereafter, the ratios labeled/unlabeled derivatives were determined by GC-C-IRMS (Finnigan MAT 252). Standard regression curves were applied in all isotopic enrichment analyses to assess for linearity of the mass spectrometer and to control for the loss of tracer. Enrichments were corrected for the presence of all isotopes of the same moiety (Biolo et al., 1992).

Calculations. Ingestion of L-[1-¹³C]phenylalanine labeled beef, intravenous infusion of L-[ring-²H₅]phenylalanine and L-[ring-²H₂]tyrosine, and arterialized blood sampling were used to assess whole-body Phe kinetics in non-steady-state conditions. Total, exogenous (Exo), and endogenous (Endo) rate of appearance (R_a) and plasma availability of dietary Phe (i.e., fraction of dietary Phe that appeared in the systemic circulation, Phe_{plasma}) were calculated using modified Steele's equations (Boirie et al., 1996; Dangin et al., 2003). These parameters were calculated as follows:

$$\text{Total } R_a = \frac{F - pV \cdot C(t) \cdot dE_{iv}/dt}{E_{iv}(t)}, \quad [1]$$

$$\text{Exo } R_a = \frac{\text{Total } R_a \cdot E_{po}(t) + pV \cdot dE_{po}/dt}{E_{prot}}, \quad [2]$$

$$\text{Endo } R_a = \text{Total } R_a - \text{Exo } R_a - F, \quad [3]$$

$$Phe_{plasma} = \left(\frac{\text{AUC}_{\text{ExoPhe}R_a}}{Phe_{Prot}} \right) \cdot 100, \quad [4]$$

where F is the intravenous tracer infusion rate (µmol/kg per min); pV (0.125) is the distribution volume for Phe (Boirie et al., 1996); $C(t)$ is the mean plasma Phe concentration between 2 time points; dE_{iv}/dt represents the time-dependent variations of plasma Phe enrichment derived from the intravenous tracer; $E_{iv}(t)$ is the mean plasma Phe enrichment from the intravenous tracer between 2 consecutive time points; $\text{Exo } R_a$ represents the plasma entry rate of dietary phenylalanine; $E_{po}(t)$ is the mean plasma Phe enrichment for the oral tracer; dE_{po}/dt represents the time-dependent variations of plasma Phe enrichment derived from the oral tracer; E_{prot} is the L-[1-¹³C]phenylalanine enrichment in the dietary protein; $\text{Endo } R_a$ represents the plasma

entry rate of phenylalanine derived from whole-body protein breakdown; Phe_{prot} is the amount of dietary Phe ingested, $AUC_{ExoPheRa}$ represents the area under the curve (AUC) of Exo Phe R_a , which corresponds to the amount of dietary Phe that appeared in the blood over a 6-h period following beef ingestion.

Total rate of disappearance of Phe (Total R_d) equals the rate of Phe hydroxylation and utilization for protein synthesis. These parameters can be calculated as follows:

$$\text{Total } R_d = \text{Total } R_a - pV \cdot \frac{dC}{dt}, \quad [5]$$

$$\text{Phe hydroxylation} = \text{Tyr } R_a \cdot \frac{E_t(t)}{E_p(t)} \cdot \frac{\text{Phe } R_d}{(F_p + \text{Phe } R_d)}, \quad [6]$$

$$\text{Protein synthesis} = \text{Total } R_d - \text{Phe hydroxylation}, \quad [7]$$

$$\text{Phe net balance} = \text{Protein synthesis} - \text{Endo } R_a, \quad [8]$$

where $E_t(t)$ and $E_p(t)$ represent the mean plasma L-[ring- $^2\text{H}_4$]tyrosine and L-[ring- $^2\text{H}_5$]phenylalanine enrichment between 2 consecutive time points, respectively.

RESULTS

Production of Intrinsically Labeled Protein

Plasma and Milk Analyses. Infusion of L-[1- ^{13}C]phenylalanine in the cow substantially increased plasma L-[1- ^{13}C]phenylalanine enrichment (Figure 2). During the infusion period, mean plasma L-[1- ^{13}C]phenylalanine enrichment averaged 37.4 ± 1.2 MPE. As a result of the L-[1- ^{13}C]phenylalanine infusion, plasma L-[1- ^{13}C]tyrosine enrichment also increased during the experiment and averaged 7.0 ± 0.4 MPE.

Milk L-[1- ^{13}C]phenylalanine and L-[1- ^{13}C]tyrosine enrichments over time are illustrated in Figure 2. Milk L-[1- ^{13}C]phenylalanine enrichment averaged 36.2 ± 0.7 and 10.3 ± 0.2 MPE in the high- and low-labeled batches, respectively. Milk L-[1- ^{13}C]tyrosine enrichment averaged 8.0 ± 0.2 and 2.4 ± 0.1 MPE, respectively. The labeled batches provided 178 and 86 kg of milk, respectively. Both batches were successfully fractionated into a high- and low-labeled casein protein concentrate (36 and 16 kg, respectively) and high- and low-labeled whey protein concentrate (15 and 13 kg, respectively). The composition of the milk protein concentrates are shown in Table 1. The total yield of intrinsically L-[1- ^{13}C]phenylalanine-labeled casein was 3.3 and 1.6 kg in the high- and low-labeled batches, respectively.

In total, 0.6 and 0.3 kg of whey protein were isolated from the high- and low-labeled batches, respectively. The produced protein concentrates met all food-grade specifications and were subsequently released for human consumption.

Of the 400 g of L-[1- ^{13}C]phenylalanine that was infused in the cow, approximately 105 g was recovered in the collected milk. Consequently, the recovery rate of tracer in milk protein was calculated to be 26%.

Meat Analyses. The mean ($\pm$ SEM) L-[1- ^{13}C]phenylalanine enrichment of the collected lean beef was 1.38 ± 0.05 MPE. The enrichment of the different meat cuts are shown in Table 2. Subjects in the human experiment ingested minced beef (from the leg), which had a L-[1- ^{13}C]phenylalanine enrichment of 1.47 ± 0.02 MPE. Based on a total body mass of 642 kg, the estimated skeletal muscle mass of our cow was approximately 200 kg. As beef contains 7.5 g of Phe per kg (Symons et al., 2007), the recovery rate of tracer in the total skeletal muscle protein pool was approximately 6%.

Human Proof-of-Principle Study

Plasma Analyses. The time course of the plasma L-[1- ^{13}C]phenylalanine and L-[ring- $^2\text{H}_5$]phenylalanine enrichments in the human experiment are illustrated in Figure 3A. Plasma L-[1- ^{13}C]phenylalanine enrichment (derived from the intrinsically labeled meat) rapidly increased following beef ingestion, reaching peak levels after 90 min (0.61 ± 0.04 MPE), after which they subsequently declined. The plasma L-[ring- $^2\text{H}_5$]phenyl-

Figure 2. Cow plasma and milk enrichments of L-[1- ^{13}C]phenylalanine and L-[1- ^{13}C]tyrosine obtained before, during, and after the infusion protocol. Values are expressed as mole percent excess (MPE).

Table 1. Composition of the milk protein concentrates

Protein concentrate	Total protein (%)	Casein and denatured whey protein (%) ¹	Native whey protein (%) ¹
High L-[1- ¹³ C] Phe-labeled casein	9.03	8.75 (97%)	0.28 (3%)
Low L-[1- ¹³ C] Phe-labeled casein	10.40	10.10 (97%)	0.31 (3%)
High L-[1- ¹³ C] Phe-labeled whey	3.81	0.28 (7%)	3.53 (93%)
Low L-[1- ¹³ C] Phe-labeled whey	2.55	0.29 (11%)	2.26 (89%)

¹The amounts of casein or whey protein in the protein concentrates are shown in parentheses. The presence of denatured whey proteins is regarded as zero because the temperature during processing was below the denaturation temperature of whey protein.

alanine enrichment decreased following the ingestion of the beef, after which levels returned toward basal values.

Whole-Body Phe Kinetics. Ingestion of the intrinsically labeled beef resulted in a rapid increase in the appearance rates of exogenous Phe, reaching peak levels after 120 min (0.42 ± 0.01 $\mu\text{mol/kg per h}$), after which they subsequently declined over time (Figure 3B). In contrast, appearance rates of endogenous Phe decreased over time following ingestion of the beef (Figure 3C). The calculated percentage of ingested Phe that appeared in the circulation was $79 \pm 1\%$. Whole-body net protein balance, calculated using plasma Phe kinetics, was -4.5 ± 0.1 $\mu\text{mol/kg per h}$ in the post-absorptive period and 6.4 ± 0.1 $\mu\text{mol/kg per h}$ in the postprandial period.

DISCUSSION

The present study shows that intrinsically L-[1-¹³C] phenylalanine-labeled milk and meat protein can be successfully produced by infusing a large amount of L-[1-¹³C]phenylalanine in a lactating dairy cow. Levels of L-[1-¹³C]phenylalanine enrichment as high as 40 MPE in milk and 1.5 MPE in meat protein were achieved. This level of milk protein enrichment is greater than any previously produced batch of intrinsically labeled milk protein. In addition, this is the first study to report the production of intrinsically labeled meat protein and show its applicability as a functional tool to assess in vivo protein digestion and absorption kinetics following beef ingestion in humans.

In the present study, we extend our previous work by investigating the potential to produce intrinsically L-[1-¹³C]phenylalanine-labeled milk protein with an enrichment level that is greater than any previously produced batch of intrinsically labeled milk protein (Boirie et al., 1995; van Loon et al., 2009; Reitelseder et al., 2011). Therefore, we applied a different regimen of L-[1-¹³C] phenylalanine isotope tracer administration to a lactating dairy cow. During tracer infusion, cow plasma L-[1-¹³C]phenylalanine enrichments reached levels as high as 37 to 44 MPE (Figure 2), values considerably higher

than the highest plasma enrichments that have been published previously (van Loon et al., 2009). The high plasma enrichments were closely matched by a comparable increase in milk L-[1-¹³C]phenylalanine enrichment during tracer infusion (Figure 2). Consequently, the absolute L-[1-¹³C]phenylalanine enrichment of the milk protein batches obtained in the current experiment was greater than previously reported and averaged 36.2 versus 29.4 and 10.3 versus 6.1 MPE, in the high- and low-labeled batches, respectively. The higher tracer enrichment could be attributed to both the longer tracer infusion (i.e., 96 vs. 48 h) as well as the co-infusion of glucose. The latter should reduce plasma amino acid oxidation and, as such, maximize the tracer incorporation in endogenous protein (Vanhatalo et al., 2003). Of course, we can only speculate on the effect of glucose co-infusion on the proposed inhibition of amino acid oxidation. As the first step in Phe oxidation is the hydroxylation to Tyr, the magnitude of this process could be estimated from the accompanying increase in L-[1-¹³C]tyrosine enrichment in both cow plasma and milk (Figure 2). The plasma L-[1-¹³C]tyrosine enrichments averaged 5.4 ± 0.6 and 7.5 ± 0.8 MPE in the previous and current experiment, respectively. The latter suggests that plasma amino acid oxidation did not differ greatly between the protocols. Therefore, the higher label enrichment of the milk batches in the present study is likely attributed to the longer tracer infusion protocol. This protocol allowed for a longer period during which highly labeled milk could be collected, which is of particular relevance as L-[1-¹³C]phenylalanine milk enrichment takes about 32 h to reach peak enrichment levels (Figure 2).

Table 2. L-[1-¹³C]Phenylalanine enrichment of different meat cuts (loin rib, shoulder, and leg) after the labeling procedure

Meat cut	Enrichment (MPE) ¹
Loin rib	1.30 ± 0.03
Shoulder	1.37 ± 0.12
Leg	1.47 ± 0.06

¹Enrichments are expressed as mole percent excess (MPE; mean $\pm$ SEM).

The recovery rate of tracer in milk protein was calculated to be 26%, which is in agreement with previous observations (Boirie et al., 1995; van Loon et al., 2009). As only 26% of the infused tracer was retrieved in the labeled milk, 74% of the tracer had been directed to other tissues and metabolic processes. We speculated that with a 4-d infusion protocol, a large amount of tracer would also have been incorporated in the muscle protein pool of the cow. Therefore, lean beef was harvested to evaluate whether this isotope tracer administration protocol would also allow the production of intrinsically labeled meat. As a result, labeled beef was obtained with a L-[1-¹³C]phenylalanine enrichment of 1.38 ± 0.05 MPE. This is the first study to report the successful production of intrinsically isotope-labeled meat. The level of enrichment is much lower than that observed in the milk protein, which is not surprising considering the relatively low protein turnover rate of skeletal muscle protein compared with the rate of milk protein production in a lactating dairy cow. However, despite a total body mass of 642 kg and an estimated skeletal muscle mass of more than 200 kg, we obtained a considerable level of muscle tissue L-[1-¹³C]phenylalanine enrichment (Table 2). Theoretically, this level of enrichment should be sufficient to allow assessment of in vivo protein digestion and absorption kinetics following beef ingestion. To confirm the applicability of the intrinsically labeled meat in studying in vivo protein digestion and absorption kinetics, a subsequent proof-of-principle experiment was performed in humans.

In the human experiment, 2 healthy young men were selected to ingest 135 g of L-[1-¹³C]phenylalanine-labeled minced beef. Immediately after ingestion of the minced beef, plasma amino acid concentrations (data not shown) and plasma L-[1-¹³C]phenylalanine enrichments (Figure 3A) increased. Calculation of the exogenous Phe appearance rate allowed for the direct assessment of dietary Phe that appeared in the circulation during the 6-h postprandial period (Figure 3B). The percentage of dietary Phe that became available in the circulation was 79%. This percentage tends to be much higher compared with label retention reported following the ingestion of milk protein (Pennings et al., 2011). Future research, therefore, should compare the bioavailability of dietary protein-derived amino acids between different protein sources. As plasma amino acid availability increased due to the digestion and absorption of the ingested meat, endogenous Phe appearance rates decreased (Figure 3C). These changes were indicative for a shift to a more anabolic situation. In fact, when subtracting whole-body protein synthesis rates from whole-body breakdown rates, it became apparent that beef ingestion strongly increased whole-body net protein balance (-4.5 ± 0.1 vs. 6.4 ± 0.1 $\mu\text{mol/kg}$

Figure 3. (A) Plasma L-[ring-²H₅]phenylalanine and L-[1-¹³C]phenylalanine enrichments expressed as mole percent excess (MPE); and (B) exogenous and (C) endogenous rates of phenylalanine appearance (expressed as $\mu\text{mol/kg}$ per min) following beef ingestion in the human experiment ($n = 2$). All values represent means $\pm$ SEM.

per h in the postabsorptive and postprandial period, respectively). Such an increase is in line with previous work that assessed whole-body net protein balance following amino acid or milk protein ingestion (Biolo et al., 1992; Boirie et al., 1996; Wagenmakers, 1999; Dangin et al., 2002, 2003; Koopman et al., 2009a,b).

The present study shows that intrinsically L-[1-¹³C] phenylalanine-labeled milk and meat protein can be successfully produced by infusing a large amount of L-[1-¹³C] phenylalanine in a lactating dairy cow. Levels of L-[1-¹³C]phenylalanine enrichment as high as 40 MPE in milk and 1.5 MPE in meat protein were achieved. This level of milk protein enrichment is greater than any previously produced batch of intrinsically labeled milk protein. In addition, this is the first study to report the production of intrinsically labeled meat protein and show its applicability as a functional tool to assess in vivo protein digestion and absorption kinetics following beef ingestion in humans. Because dairy and meat are considered high quality and widely consumed protein sources, the applicability of intrinsically labeled milk and meat protein will be instrumental to obtain further insight in human nutrition research.

ACKNOWLEDGMENTS

The suggestions and help of Ger de Vries-Reijling (Adaptation Physiology Group, Wageningen University) and Inge van Geijlswijk (Veterinary Pharmacy, Utrecht University) to prepare the infusates are highly appreciated, as is true for the discussions with Rob Steenmans (advisor to the committee on the use of experimental animals at Wageningen University) to optimize the experimental conditions. The expert technical assistance of Marleen Scheer, Daniel Warner, Alexandre Carlu, and the staff of the experimental farm "De Ossekampen" is greatly appreciated.

REFERENCES

- Abumrad, N. N., D. Rabin, M. P. Diamond, and W. W. Lacy. 1981. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. *Metabolism* 30:936–940.
- Berthold, H. K., D. L. Hachey, P. J. Reeds, O. P. Thomas, S. Hoeksema, and P. D. Klein. 1991. Uniformly ¹³C-labeled algal protein used to determine amino acid essentiality in vivo. *Proc. Natl. Acad. Sci. USA* 88:8091–8095.
- Biolo, G., P. Tessari, S. Inchiostro, D. Bruttomesso, C. Fongher, L. Sabadin, M. G. Fratton, A. Valerio, and A. Tiengo. 1992. Leucine and phenylalanine kinetics during mixed meal ingestion: A multiple tracer approach. *Am. J. Physiol.* 262:E455–E463.
- Boirie, Y., J. Fauquant, H. Rulquin, J. L. Maubois, and B. Beaufre. 1995. Production of large amounts of [¹³C]leucine-enriched milk proteins by lactating cows. *J. Nutr.* 125:92–98.
- Boirie, Y., P. Gachon, S. Corny, J. Fauquant, J. L. Maubois, and B. Beaufre. 1996. Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. *Am. J. Physiol.* 271:E1083–E1091.
- Dangin, M., Y. Boirie, C. Guillet, and B. Beaufre. 2002. Influence of the protein digestion rate on protein turnover in young and elderly subjects. *J. Nutr.* 132:3228S–3233S.
- Dangin, M., C. Guillet, C. Garcia-Rodenas, P. Gachon, C. Bouteloup-Demange, K. Reiffers-Magnani, J. Fauquant, O. Ballevre, and B. Beaufre. 2003. The rate of protein digestion affects protein gain differently during aging in humans. *J. Physiol.* 549:635–644.
- Deglaire, A., P. J. Moughan, C. Bos, K. Petzke, S. M. Rutherford, and D. Tome. 2008. A casein hydrolysate does not enhance gut endogenous protein flows compared with intact casein when fed to growing rats. *J. Nutr.* 138:556–561.
- Evenepoel, P., M. Hiele, A. Luybaerts, B. Geypens, J. Buyse, E. Decuyper, P. Rutgeerts, and Y. Ghos. 1997. Production of egg proteins, enriched with L-leucine-¹³C1, for the study of protein assimilation in humans using the breath test technique. *J. Nutr.* 127:327–331.
- Husek, P. 1991. Amino acid derivatization and analysis in five minutes. *FEBS Lett.* 280:354–356.
- Koopman, R., N. Crombach, A. P. Gijzen, S. Walrand, J. Fauquant, A. K. Kies, S. Lemosquet, W. H. Saris, Y. Boirie, and L. J. van Loon. 2009a. Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein. *Am. J. Clin. Nutr.* 90:106–115.
- Koopman, R., W. H. Saris, A. J. Wagenmakers, and L. J. van Loon. 2007. Nutritional interventions to promote post-exercise muscle protein synthesis. *Sports Med.* 37:895–906.
- Koopman, R., S. Walrand, M. Beelen, A. P. Gijzen, A. K. Kies, Y. Boirie, W. H. Saris, and L. J. van Loon. 2009b. Dietary protein digestion and absorption rates and the subsequent postprandial muscle protein synthetic response do not differ between young and elderly men. *J. Nutr.* 139:1707–1713.
- Pennings, B., Y. Boirie, J. M. Senden, A. P. Gijzen, H. Kuipers, and L. J. van Loon. 2011. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am. J. Clin. Nutr.* 93:997–1005.
- Reitelseder, S., J. Agergaard, S. Doessing, I. C. Helmark, P. Lund, N. B. Kristensen, J. Frystyk, A. Flyvbjerg, P. Schjerling, G. van Hall, M. Kjaer, and L. Holm. 2011. Whey and casein labeled with L-[1-¹³C]leucine and muscle protein synthesis: effect of resistance exercise and protein ingestion. *Am. J. Physiol. Endocrinol. Metab.* 300:E231–E242.
- Schei, I., A. Danfaer, I. A. Boman, and H. Volden. 2007. Post-ruminal or intravenous infusions of carbohydrates or amino acids to dairy cows. 1. Early lactation. *Animal* 1:501–514.
- Symons, T. B., S. E. Schutzler, T. L. Cocke, D. L. Chinkes, R. R. Wolfe, and D. Paddon-Jones. 2007. Aging does not impair the anabolic response to a protein-rich meal. *Am. J. Clin. Nutr.* 86:451–456.
- Tamminga, S., W. M. Van Straalen, A. P. J. Subnel, R. G. M. Meijer, A. Steg, C. J. G. Wever, and M. C. Blok. 1994. The Dutch protein evaluation system: The DVE/OEB-system. *Livest. Prod. Sci.* XX:139–155.
- van Eijk, H. M., D. R. Rooyackers, and N. E. Deutz. 1993. Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2–3 microns Spherisorb ODS II column. *J. Chromatogr.* 620:143–148.
- Van Es, A. J. H. 1978. Feed evaluation for ruminants. I. The systems in use from May 1977 onwards in The Netherlands. *Livest. Prod. Sci.* 5:331–345.
- van Loon, L. J., Y. Boirie, A. P. Gijzen, J. Fauquant, A. L. de Roos, A. K. Kies, S. Lemosquet, W. H. Saris, and R. Koopman. 2009. The production of intrinsically labeled milk protein provides a functional tool for human nutrition research. *J. Dairy Sci.* 92:4812–4822.
- Vanhatalo, A., T. Varvikko, and P. Huhtanen. 2003. Effects of casein and glucose on responses of cows fed diets based on restrictively fermented grass silage. *J. Dairy Sci.* 86:3260–3270.
- Wagenmakers, A. J. 1999. Tracers to investigate protein and amino acid metabolism in human subjects. *Proc. Nutr. Soc.* 58:987–1000.
- Wolfe, R. R., and D. L. Chinkes. 2005. *Isotope Tracers in Metabolic Research*. Wiley-Liss, New York, NY.