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UMR Physiologie de la Nutrition du Comportement Alimentaire, AgroParisTech, Institut National de la Recherche Agronomique, Université Paris Saclay, Paris, France

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Stepien M, Azzout-Marniche D, Even PC, Khodorova N, Fromentin G, Tomé D, Gaudichon C. Adaptation to a high-protein diet progressively increases the postprandial accumulation of carbon skeletons from dietary amino acids in rats. *Am J Physiol Regul Integr Comp Physiol* 311: R771–R778, 2016. First published August 31, 2016; doi:10.1152/ajpregu.00040.2016.—We aimed to determine whether oxidative pathways adapt to the overproduction of carbon skeletons resulting from the progressive activation of amino acid (AA) deamination and ureagenesis under a high-protein (HP) diet. Ninety-four male Wistar rats, of which 54 were implanted with a permanent jugular catheter, were fed a normal protein diet for 1 wk and were then switched to an HP diet for 1, 3, 6, or 14 days. On the experimental day, they were given their meal containing a mixture of 20 U-[^{15}N]-[^{13}C] AA, whose metabolic fate was followed for 4 h. Gastric emptying tended to be slower during the first 3 days of adaptation. ^{15}N excretion in urine increased progressively during the first 6 days, reaching 29% of ingested protein. $^{13}CO_2$ excretion was maximal, as early as the first day, and represented only 16% of the ingested proteins. Consequently, the amount of carbon skeletons remaining in the metabolic pools 4 h after the meal ingestion progressively increased to 42% of the deaminated dietary AA after 6 days of HP diet. In contrast, ^{13}C enrichment of plasma glucose tended to increase from 1 to 14 days of the HP diet. We conclude that there is no oxidative adaptation in the early postprandial period to an excess of carbon skeletons resulting from AA deamination in HP diets. This leads to an increase in the postprandial accumulation of carbon skeletons throughout the adaptation to an HP diet, which can contribute to the sustainable satiating effect of this diet.

dietary amino acids; oxidation; deamination; glycogen; stable isotopes

THE EFFECT OF HIGH-PROTEIN (HP) diets on energy metabolism has been often questioned (15, 29, 43). In rats fed ad libitum, an HP diet has been shown to lower adiposity, an effect that is not only due to a reduction in the spontaneous energy intake, as revealed by pair-fed studies (4, 30), but also due to metabolic adaptations. When dietary proteins are given in excess, the metabolic pathways adapt in a few days to cope with the excess nitrogen originating from amino acids through an activation of various liver enzymes involved in their catabolism. This has been well documented for the urea cycle (25, 33). In contrast, less is known about the remaining carbon skeletons. It seems that the carbon skeletons produced from the increased deamination are poorly catabolized during the postprandial window (14). Indeed, using ^{15}N and ^{13}C amino acids administered as oral tracers in an HP meal given to rats, we found that 4 h after ingestion almost half of the carbon skeletons produced from dietary amino acids were still not oxidized (14).

Minimal data exist on the adaptation of energy pathways to a large increase in dietary protein intake. In a previous study, we examined the time course adaptation of energy metabolism components to a high-protein diet in rats (38). Together with the adaptation of key enzymes involved in energy pathways (39), we reported a progressive adaptation of glucose metabolism over 2 wk, characterized by an increase in carbohydrate (CHO) oxidation above CHO intake. In contrast, we found no significant changes in energy expenditure.

A better understanding of the channeling of carbon skeletons from dietary amino acids toward metabolic pathways is of importance to better understand how amino acids contribute to energy metabolism in situations of protein excess. To compare the time course adaptation of dietary nitrogen and carbon skeleton metabolism to an HP diet, we used a double stable isotope method (^{15}N and ^{13}C) to trace dietary proteins and determine the metabolic fate of the nitrogen and carbon compounds.

MATERIALS AND METHODS

Animals and Diets

All experiments were carried out in accordance with the guidelines of the French Committee for Animal Care (agreement number: 75–1562) and the European Convention on Vertebrate Animals used for Experimentation. Male Wistar rats (160–180 g) were purchased from Harlan (Horst, The Netherlands) and housed under controlled environmental conditions (temperature: 21°C ± 1, 12:12-h reversed light-dark cycle, lights off at 0900). The rats had free access to water and a normal protein diet (NP; 14% protein as energy) (Table 1) for 5 days.

After the habituation period, a group of rats underwent a surgical procedure to have implanted a permanent jugular catheter, according to a technique previously described (27, 38). Rats were anesthetized with an intraperitoneal injection of xylazine and ketamine (10 and 75 mg/kg, respectively). After a superficial skin incision at the jugular level, a catheter (Silastic, external diameter = 1.19 mm; internal diameter = 0.63 mm) was inserted in the external jugular vein and pushed down the vena cava. The external part of the catheter was pulled subcutaneously to a slit in the skin and fixed on the top of the skull with dental cement (Dentalon). Sixty-four rats were operated and three of them died during the surgery or the day after. During 1 wk after the surgery, rats received prophylaxis to ensure analgesia and to prevent infection until complete recovery. During this recovery period, all rats were fed the NP diet. At the end of this period, 11 rats were kept on the NP diet, whereas the remaining rats (n = 43) were fed an HP diet (55% protein as energy; Table 1) for periods of different lengths: 1, 3, 6, and 14 days. They are hereon referred to as groups HP1 (n = 10), HP3 (n = 8), HP6 (n = 14), and HP14 (n = 11), respectively. The unequal number of rats in the experimental

Address for reprint requests and other correspondence: C. Gaudichon, UMR914 INRA-AgroParisTech Nutrition Physiology and Ingestive Behavior, AgroParisTech, 16 rue Claude Bernard, F-75005 Paris, France (e-mail: Claire.Gaudichon@agroparistech.fr.).

Table 1. *Diet composition*

Nutrient, g/kg	Normal Protein	High Protein
Total milk protein[1]	140	530
Pregelatinized cornstarch[2]	622.4	277
Sucrose[3]	100.3	50
Soybean oil[4]	40	40
Mineral mixture[5] (AIN93-M)	35	35
Vitamin mixture[5] (AIN93-V)	10	10
Cellulose[6]	50	50
Choline[5]	2.3	2.3
Total	1.000	1.000
Energy, kJ/g	14.6	14.6

[1]IDI;Arras, France; [2]Cerestar, Haubourdin, France; [3]Eurosucre, Paris, France; [4]Bailly SA, Aulnay-sous-bois, France; [5]ICN Biochemicals, OH (see Reeves et al. 1993 for composition); [6]Medias filtrants Durieux, Torcy, France. P/E, G/E, L/E: percentage of diet energy provided by protein, carbohydrates and lipids, respectively.

groups was due to the fact that not all of the implanted catheters remained permeable, which prevented blood sampling and/or infusions in seven rats.

Finally, 54 rats implanted with the venous catheters underwent the complete 4-h metabolic tracer study. In addition, 30 nonoperated rats were used to determine gastrointestinal contents 2 h after the meal, and 10 more rats were used to determine the natural isotopic enrichments in the organs (Fig. 1).

Every day, the rats received the experimental diet in two periods: one calibrated meal of 4 g (58 kJ) given between 0900 and 0930 and free access to food between 1200 and 1800. The morning calibrated meal was given to train the rats to quickly consume a standardized amount of food. It contained 0.56 g and 2.12 g of protein for NP and HP conditions, respectively.

Experimental Procedure

Complete metabolic tracer study. On the last day of dietary adaptation, at 1800, the operated animals were individually placed in a 9-liter open-circuit indirect calorimetric chamber that included a food cup (11), with no access to food as usual during the light period. At 0930 on the next day, they were given the appropriate meal of 4 g, moistened with 4 ml of water to prevent spillage, and enriched with 6 mg of a mixture of 20 amino acids, nitrogen, and carbon being uniformly labeled (^{15}N, 98%; ^{13}C, 98%) (CortectNet, Paris, France).

The catheter was extended by a Silastic tubing fixed to a balanced arm at the top of the cage, according to the method described by Nicolaidis et al. (27). Throughout the study, the rats were infused with hypotonic saline (4‰) at a rate of 6 ml/h, and urine was collected at 30-min intervals using a fraction collector with tubes containing 20 μl 0.1 N HCl to prevent bacterial proliferation (12). During the 4-h postprandial period, expired air was collected from the cage outflow in glass Vacutainers every 0.5 h, and plasma was sampled through the permanent catheter every 0.5 h for 2 h and then hourly for the next 2 h. Plasma was kept at −20°C until determination of tracers in plasma protein and glucose. Glucose was immediately assayed using a standard glucometer. The volume of urine collected in each tube was measured, and samples were aliquoted and frozen at −20°C for subsequent determinations of ^{15}N in urea.

Four hours after the beginning of the meal (i.e., at 1330), animals were killed with a pentobarbital bolus through the catheter (40 mg/kg). The rats were then exsanguinated, and the blood was centrifuged and aliquoted for urea analysis. The livers were collected, weighed, and quickly frozen in liquid nitrogen. They were then kept at −20°C for glycogen analysis. The stomachs and small intestines were removed, and their contents were collected for further determination of dietary N content.

Ten nonoperated control rats were given an experimental meal without any tracer and were killed 4 h after the meal. The digestive contents, liver, urine and blood were collected for further determination of natural enrichments.

Complementary digestive study. Thirty additional nonoperated rats were fed a labeled meal and were killed 2 h after ingestion to determine the digestive contents at this intermediary time point.

Analytical Methods

Urinary and plasma urea concentrations were determined using a commercial kit (Bio-Mérieux, Marcy l'Etoile, France). Plasma urea was extracted by cation exchange chromatography on Dowex resin (AG 50W-X8 resin 100–200 Mesh Na form; Bio-Rad, Hercules, CA), as previously described (19), and stored at 4°C until isotopic determination. Liver glycogen content was determined as the difference between the glucose content before and after glycogen hydrolysis with amyloglucosidase, as previously described (14). For plasma glucose isotopic determinations, glucose was purified, as described previously (14, 21).

^{15}N and ^{13}C enrichments in urinary and plasma urea were determined using an isotopic ratio mass spectrometer (IRMS, Isoprime, GV Instrument, Manchester, UK) coupled to an elemental analyzer (EA) (Euro Elemental Analyser 3000, EuroVector). ^{13}C enrichment in hepatic glycogen and plasma glucose was measured in six rats per group using gas chromatography-combustion-IRMS analysis, after derivatization with methyl boronic acid, as described elsewhere (21). For plasma glucose, the enrichments were determined only in the NP, HP1, and HP14 groups. The ^{13}C enrichment of CO_2 in expired gas was determined using GC-isotope-ratio MS (Multiflow/Isoprime, Mi-

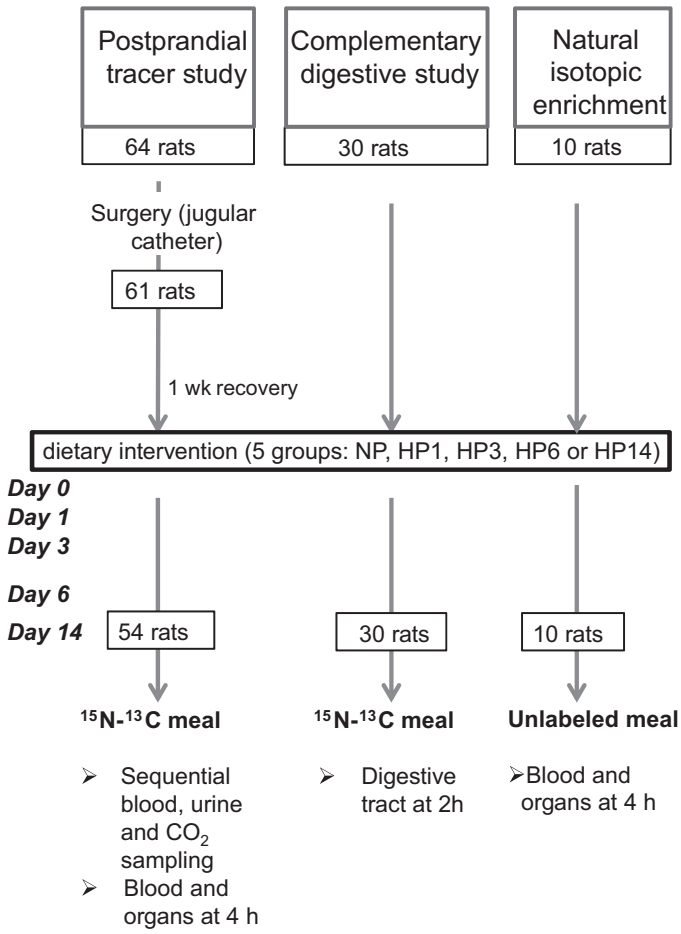

Fig. 1. Overview of experimental design.

cromass). Total nitrogen and carbon in the gastrointestinal contents were measured using the EA, with atropine (CE Instruments, Milan, Italy) and cyclohexanone (CE Instruments), respectively, as standards.

Calculations

Dietary amino acid transfer in digestive and metabolic pools. The dietary AA present in the samples were expressed in grams of dietary proteins and were calculated as follows. For the nitrogen part: Dietary protein = $N_{tot} \times 14 \times 6.25 \times (APE_{sample}/APE_{meal})$, where N_{tot} is the amount of total nitrogen (mmol) in the sample; APE_{sample} is the ^{15}N enrichment percent excess of the sample = enrichment of the sample − basal enrichment, in atom percent (AP); and APE_{meal} is the ^{15}N enrichment percent excess of the meal = enrichment of the meal − basal enrichment.

The conversion factor from N to protein was assumed to be 6.25 (16% of N in proteins).

The ^{15}N enrichment of the meal (in AP) was 1.28% and 0.6% in the NP and HP diets, respectively. The difference between meal enrichments was of similar amplitude to that obtained in previous metabolic studies that compared the postprandial fate of intrinsic labeled soy and milk proteins (5, 22). For the carbon part: Dietary protein = $C_{tot} \times 12 \times 2 \times (APE_{sample}/APE_{meal})$, where C_{tot} is the amount of total carbon (mmol) in the sample and APE_{sample} is the ^{13}C enrichment percent excess of the sample, which is equal to the enrichment of the sample − basal enrichment.

APE_{meal}. ^{13}C enrichment percent excess of the meal = enrichment of the meal − basal enrichment. The conversion factor from C to protein was assumed to be 2 (50% of C in proteins).

The ^{13}C enrichment of the meal was 2.01% and 1.33% in the NP and HP meals, respectively.

Total absorption of dietary amino acids. The percentage of dietary amino acids absorbed 4 h after meal ingestion was calculated as the difference between ingested proteins and the amount of dietary proteins recovered 4 h after meal ingestion in the stomach and small intestine.

Postprandial deamination and oxidation of dietary amino acids. The recovery of dietary AA in the urea body pool 4 h after the meal, expressed in grams of dietary protein, was calculated according to the following formula: $N_{exo\text{-}urea}$ = [urea] × 2 × 0.67/0.92 × BW × (APE_{urea}/APE_{meal}), where [urea] is the concentration of urea in the plasma (mmol/l) and BW is the body weight in grams.

The mean percentages of body water and water in plasma of the rats were assumed to be 67% and 92%, respectively (19, 23).

Finally, total deamination of dietary amino acids was calculated as the sum of the dietary protein recovered in urinary urea and the body urea pool during the 4 h after the meal.

The carbon from dietary proteins recovered in expired air was calculated by combining $^{13}CO_2$ enrichment and CO_2 production, as follows: Dietary carbon recovery = $[(CO_2/22.4) \times 2 \times 12 \times (APE_{expired\ air}/APE_{meal})]$, where CO_2 is expressed in milliliters per minute. The conversion factor from C to protein was assumed to be 2 (50% of C in proteins).

Finally, the total oxidation of dietary amino acids was calculated as the sum of dietary carbon recovered in expired air at each time point, as described below: Total oxidation = $\sum_{t=0}^{4} \sum_{t}^{t+30}$ dietary carbon recovery.

Macronutrient balance. Substrate oxidation was calculated using the classical stochiometric formulas: Carbohydrate oxidation: CHOox (W) = $(4.57\ \dot{V}CO_2 - 3.23\ \dot{V}O_2 - 2.6\ N) \times (3.74 \times 4.18/60)$; Lipid oxidation: Lox (W) = $(1.69\ \dot{V}O_2 - 1.69\ \dot{V}CO_2 - 2.06\ N) \times (9.46 \times 4.18/60)$; and protein oxidation: Pox (W) = $6.25 \times N$ (mg) × $(4.32 \times 4.18/60)$, with $\dot{V}O_2$ and $\dot{V}CO_2$ in milliliters per minute, 3.74 = kcal/g for glucose, 9.46 = kcal/g for lipids, and 4.32 kcal/g for protein, as described in detail previously (11).

Four-hour macronutrient balance was calculated as the difference between macronutrient ingestion and 4-h substrate oxidation.

Statistics

Results are expressed as means ± SE. Differences between groups were tested using one-way ANOVA and post hoc Tukey tests when appropriate, using the GLM procedure under SAS (version 9.1; SAS, Cary, NC). Kinetic analyses were performed using the mixed procedure under SAS, with time as a repeated factor. Differences were considered significant for P values < 0.05.

RESULTS

Digestion Kinetics

The amount of protein actually ingested was 0.51 ± 0.03 g in the NP group. It was 1.76 ± 0.1 g in the HP1 group and then reached 1.90 to 1.93 ± 0.02 g in the other HP groups. There was no significant difference between HP groups.

The dietary proteins remaining in the stomach and the intestine 2 and 4 h after the meal were similar whether assessed for ^{15}N or ^{13}C. Two hours after the meal, there was a significant effect of the group with four- to five-fold larger amounts of protein remaining in the stomach in HP compared with NP fed rats (Fig. 2). The amount of dietary protein in the stomach also tended to decrease in HP14 rats compared with HP3 ($P = 0.07$). Four hours after the meal, the amount of protein remaining in the stomach was 9.4 ± 2.3% of ingested protein in the NP group and from 12.7 ± 3.3 to 15.6 ± 1.4% in the HP groups without any differences between HP groups, whereas NP differed from all HP groups ($P < 0.001$).

The amount of dietary proteins found in the intestine was larger in the HP groups than in the NP group, both at 2 and 4 h (not shown). There was no significant difference between HP groups, except between HP3 and HP6 at 2 h ($P = 0.05$). Finally, the amount of dietary protein absorbed after 4 h was not significantly different between the HP groups (1.58 ± 0.1 g

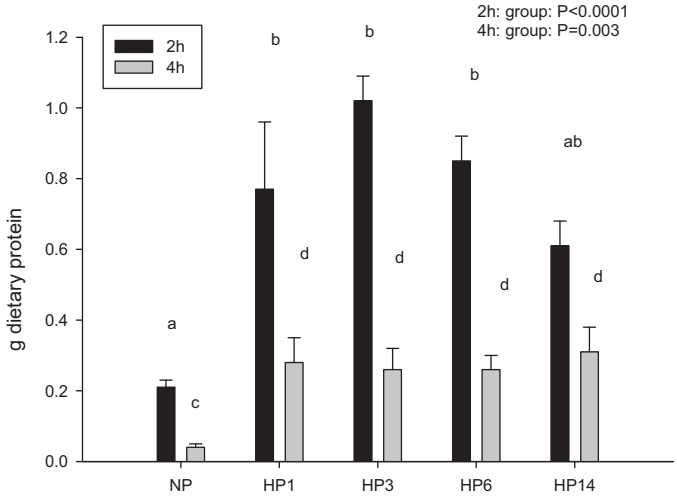

Fig. 2. Gastric content of dietary nitrogen 2 and 4 h after the ingestion of an normal protein (NP) or high protein (HP) meal containing 6 mg U-[^{15}N]-[^{13}C]-labeled AA. Rats were fed the NP control diet (NP, $n = 11$) or switched to the HP diet for 1 (HP1, $n = 10$), 3 (HP3, $n = 8$), 6 (HP6, $n = 14$), and 14 days (HP14, $n = 11$). Measurements at 4 h were obtained in the main lot of 54 rats that completed the whole study, and measurements at 2 h were obtained in a complementary lot of 30 rats ($n = 6$ per time point). Values are expressed as means ± SE. The adaptation effect was analyzed within each lot of rats (2 or 4 h) using a one-way ANOVA. Letters that differ indicate a significant difference between groups ($P < 0.05$, post hoc Tukey tests).

of proteins in HP1 and 1.75 ± 0.03 g in the other groups) and was significantly larger than in the NP group (0.3 ± 0.05 g).

Postprandial Macronutrient Balance

The postprandial balance was calculated in the 4-h postprandial period (Table 2). The CHO balance was not significantly different between NP and HP1 groups, but became negative after 3 days of the HP diet, and the protein balance was positive in all rats. The protein balance was significantly higher in all HP groups compared with the NP group. Fat balance was negative in HP1 rats and was significantly different from the other groups.

Deamination and Oxidation

The cumulated excretion of dietary nitrogen in urines (Fig. 3*A*) showed a progressive increase from 1 to 6 days and a stabilization thereafter, suggesting that full adaptation to the HP diet required no more than 6 days. Differences between the HP groups appeared after 3 h postingestion. After 1 day of the HP diet, the cumulated excretion of dietary N corresponded to only 187 ± 21 mg of dietary protein. It increased to 272 ± 41 mg after 3 days and reached 352 ± 24 mg after 6 days, representing 10.7 ± 1.2% for HP1 to 19.5 ± 2.2% for HP14 of ingested proteins. The excretion was similar between HP6 and HP14. In the NP group, ^{15}N excretion was very low and amounted to only 5.7 ± 0.9% of ingested proteins.

In NP rats, there was still 3.9 ± 0.9% of dietary N sequestered in body urea 4 h after the meal (not shown). It ranged from 6.8% ± 1 (HP1) to 8.4% ± 0.4 (HP6) in HP rats, with no difference between HP groups.

The cumulated excretion of CO_2 from dietary AA (Fig. 3*B*) was significantly higher in all HP groups than in the NP group and was not different between HP groups. Four hours after the meal, the amount of ^{13}C lost through oxidation represented 47 mg of dietary amino acids in the NP group, and 200 to 250 mg in the HP groups as soon as the first day.

The losses of dietary amino acids through deamination (i.e., the sum of ^{15}N recovery in urine and body urea) and oxidation 4 h after the meal are shown in Fig. 4. In NP rats, deamination and oxidation were similar, while in HP rats deamination was larger than oxidation, this difference being significant as early as 3 days. After 6 to 14 days, about 29% of the dietary AA were deaminated, whereas only 16% were used in the oxidative pathways and, therefore, 40% of the deaminated dietary amino acids was not oxidized.

Table 2. *Energy macronutrient balance during 4 h after the meal*

	CHO	Fat	Protein
NP	0.07 ± 0.07^{a}	0.06 ± 0.04^{a}	0.32 ± 0.03^{a,***}
HP1	−0.29 ± 0.13^{b}	−0.14 ± 0.03^{b,***}	1.22 ± 0.081.4^{b,***}
HP3	−0.48 ± 0.071.4^{b,***}	−0.003 ± 0.04^{a}	1.16 ± 0.11^{b,***}
HP6	−0.28 ± 0.09^{b,**}	0.07 ± 0.04^{a}	1.17 ± 0.09^{b,***}
HP14	−0.43 ± 0.07^{b,***}	0.05 ± 0.04^{a}	1.11 ± 0.06^{b,***}
Group effect	$P = 0.0012$	$P = 0.01$	$P < 0.0001$

Values are expressed as means ± SE [Normal Protein (NP), $n = 11$; High Protein 1 (HP1), $n = 10$; HP3, $n = 8$, HP6, $n = 14$; HP14, $n = 11$]. One-way ANOVA with group as a factor. Values with letters that differ within a column are significantly different (post-hoc Tukey test). Asterisks indicate when values are significantly different from 0 (**$P < 0.01$, ***$P < 0.005$).

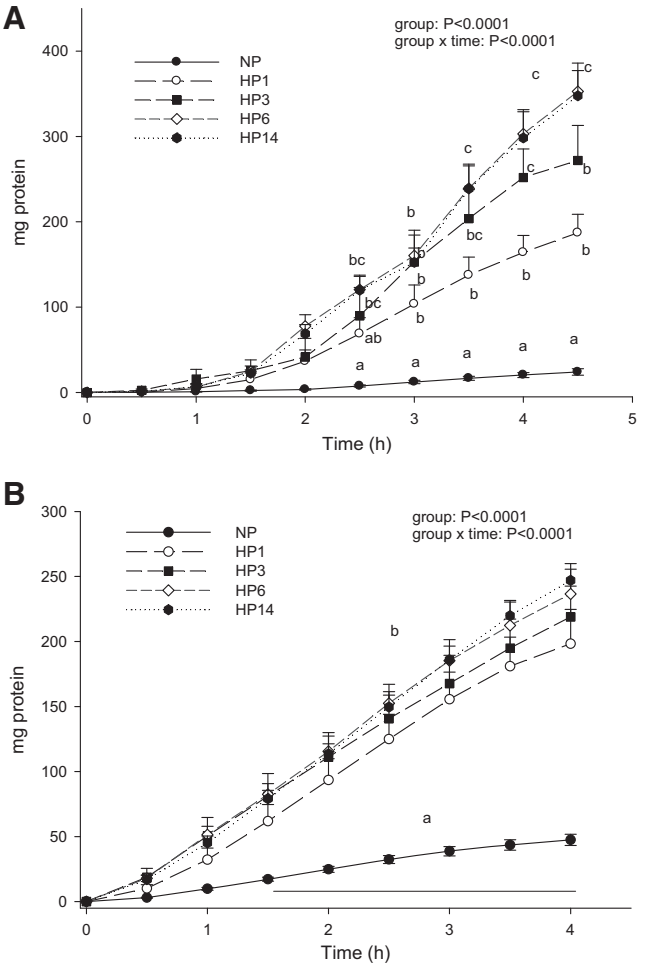

Fig. 3. Cumulative excretion of dietary nitrogen in the urine (*A*) and cumulative excretion of carbon skeletons from dietary amino acids in expired air (*B*) during 4 h after the ingestion of an NP or HP meal containing 6 mg U-[^{15}N]-[^{13}C]-labeled amino acids (AA). Rats were fed the NP control diet (NP, $n = 11$) or switched to the HP diet for 1 (HP1, $n = 10$), 3 (HP3, $n = 8$), 6 (HP6, $n = 14$), and 14 days (HP14, $n = 11$). Values are expressed as means ± SE. The adaptation effect was analyzed in a mixed model with the group as the fixed factor and time as a repeated factor within rats. Different letters indicate a significant difference between groups at each time point ($P < 0.05$, post hoc Tukey tests).

Hepatic Glycogen and Transfer of Dietary ^{13}C to Liver Glycogen

Liver glycogen was higher in the NP group than in all HP groups with no differences among HP groups (Table 3). The transfer of dietary ^{13}C to liver glycogen ($APE_{glycogen}/APE_{meal}$) was the lowest for NP rats and increased from the 1st to the 14th day of adaptation to the HP diet but without reaching significance. The same was observed when the results were expressed as a percentage of dietary tracer or in grams of dietary proteins transferred to glycogen (Table 3).

The enrichment of ^{13}C in glucose was determined in NP rats, and after 1 and 14 days of adaptation to the HP diet (Fig. 5). Glucose enrichment in HP1 and HP14 rats was significantly higher than in NP rats ($P < 0.0001$). There was also a trend for an increase in ^{13}C enrichment in glucose in HP14 rats compared with HP1 ($P = 0.09$).

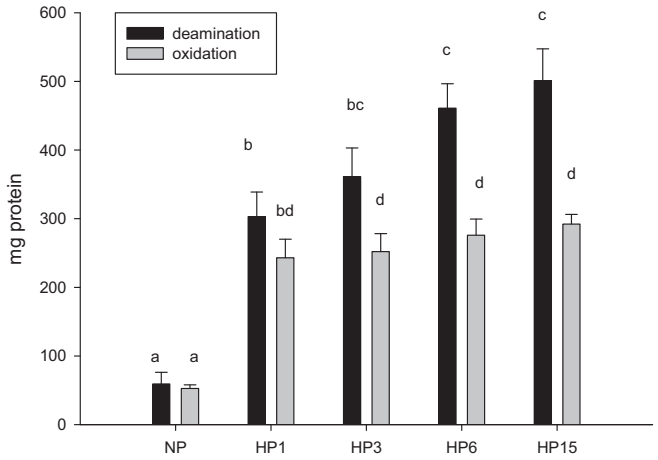

Fig. 4. Total losses of dietary amino acids in deamination and oxidative pathways during 4 h after the ingestion of an NP or HP meal containing 6 mg U-[^{15}N]-[^{13}C]-labeled AA. Rats were fed the NP control diet (NP, $n = 11$) or switched to the HP diet for 1 (HP1, $n = 10$), 3 (HP3, $n = 8$), 6 (HP6, $n = 14$), and 14 days (HP14, $n = 11$). Values are expressed as means ± SE. The adaptation effect was analyzed using a one-way ANOVA. Letters that differ indicate a significant difference between groups ($P < 0.05$, post hoc Tukey tests).

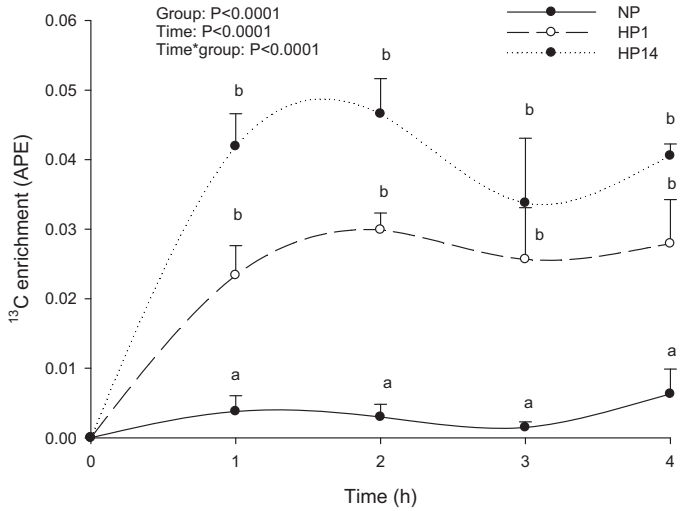

Fig. 5. ^{13}C enrichment of glucose during 4 h after the ingestion of an NP or HP meal containing 6 mg U-[^{15}N]-[^{13}C]-labeled AA. Rats were fed the NP control diet (NP) or switched to the HP diet for 1 and 14 days ($n = 6$ per group). Values are expressed as means ± SE. The adaptation effect was analyzed in a mixed model with the group as the fixed factor and time as a repeated factor within rats. Letters that differ indicate a significant difference between groups at each time point ($P < 0.05$, post hoc Tukey tests).

DISCUSSION

This study addressed the time course of metabolic adaptation to a high-protein diet, to cope with an excess of carbon skeletons originating from amino acid deamination. We discriminated the metabolic fate of α-amino groups and carbon skeletons of dietary amino acids using a double labeling of dietary proteins with ^{15}N and ^{13}C amino acids. We found a difference between the time course adaptation of nitrogen and carbon skeleton metabolisms. While carbon skeleton excretion through oxidation was maximal and saturated as soon as the first day, it required 6 days before nitrogen excretion from dietary amino acids reached its maximum. There was more deamination than oxidation as early as 3 days of the HP diet, resulting in an excess of carbon skeletons retained in the intermediary pools during the 4 h following meal ingestion.

As expected, we observed a marked increase in deamination of dietary amino acids in response to HP feeding, as illustrated by the increase in ^{15}N transfer to body and urinary urea. The adaptation of the deamination processes required 6 days before reaching its maximum, which is consistent with previous studies (23, 28). Liver urea cycle enzymes (44), and probably intestinal catabolism (40), are activated as a function of the level of circulating amino acids. Protein synthesis pathways are only moderately involved in the handling of amino acid excess (24), as the mTOR pathway was shown to be activated in response to a high amino acid level in the liver (8, 9), but not in the muscle (13). As a consequence, amino acid catabolism is increased in both the postprandial and the fed state (38), thus permitting a rapid handling of amino acids after absorption. However, the oxidation rate of labeled amino acids, obtained from breath test analyses, did show an increase as early as the first day of the HP diet, but at a level insufficient to handle the increase in deamination. This resulted in a difference between ^{15}N and ^{13}C excretion that was more marked as deamination adapted. We have already reported such a discrepancy between nitrogen and carbon excretion in the end products of rats fed an HP diet for 14 days (14). The present study further reveals that the TCA cycle probably immediately handles part of the excess carbon skeletons provided by the deamination of the dietary amino acids, but we observed no further adaptation that would permit adjusting oxidation to the increased flow of carbon skeletons. This result, as previously pointed out by Jungas et al. (17), confirms that the liver is not able to oxidize all the carbon skeletons produced from amino acids in the context of an HP intake. In the case of lipids and carbohydrates, it was also observed that in response to overfeeding, oxidation was moderately enhanced, carbohydrate oxidation increased at the expense of fat oxidation (36) and substrates were, therefore, channeled mainly toward fat storage (6, 36, 41). In contrast to the adaptation to excess fat and carbohydrate intake, there is no storage of the carbon skeletons from proteins in adipose tissues. Indeed, we verified the absence of any ^{13}C enrichment above the baseline in subcutaneous and mesenteric adipose tissues (not shown).

Table 3. *Liver glycogen and transfer of ^{13}C to glycogen*

	Liver Glycogen, mg/g liver	Glycogen Enrichment, APE	^{13}C Recovery, mg eq protein
NP	18.93 ± 8.5^{a}	0.027 ± 0.024	2.21 ± 2.16
HP1	5.93 ± 1.8^{b}	0.045 ± 0.016	4.06 ± 2.64
HP3	8.83 ± 7.0^{b}	0.051 ± 0.026	6.65 ± 6.93
HP6	7.40 ± 4.0^{b}	0.063 ± 0.022	7.69 ± 4.53
HP14	4.35 ± 3.9^{b}	0.058 ± 0.022	4.72 ± 5.46
Group effect	$P = 0.006$	NS	NS

Values are expressed as means ± SE ($n = 6$ per group). One-way ANOVA with group as factor. Values sharing different letters within a column are different (post hoc Tukey test).

Other possible fates would be the partial conversion of carbon skeletons to glucose and to ketone bodies, and/or storage as glycogen. We found a trend for a progressive increase of ^{13}C enrichment in plasma glucose from 1 to 15 days. This measurement was taken in a limited number of

animals due to the insufficient amounts of blood we could collect kinetically in some rats. Nevertheless, this trend suggests a gradual upregulation of glucose production from carbon skeletons from dietary amino acids. Consistently, gluconeogenesis has been proposed as the process of surplus amino acid disposal in HP diet conditions (17), and as the way to maintain glucose body homeostasis under these conditions (2). Previous studies have also shown that in the fed state, after adaptation to an HP diet for 2 wk, phosphoenylpyruvate carboxykinase (PEPCK), but not glucose-6-phosphatase (G6PC1), gene expression is increased (1, 39), which led us to suggest the existence of a direct production of glycogen from amino acid precursors (1). Interestingly, Rossetti et al. (35) has shown that an HP diet increases the indirect pathway of glycogen synthesis in rats. However, we did not further confirm the hypothesis that liver glycogen was involved in the storage of amino acid carbon skeletons, neither in the present study nor in the study of Fromentin et al. (14), as we observed that in HP-fed rats, liver glycogen content was decreased, and ^{13}C enrichment was not increased. These results suggest that glucose or endogenous amino acids are the substrate of glycogenogenesis, rather than dietary amino acids. Although hepatic glucose uptake was not measured in the present study, it can be assessed from a previous study in which we measured plasma glucose concentrations in the vena cava, portal vein, and hepatic artery 2 h after the meal (39). Fractional hepatic glucose uptake averaged 14% in NP rats and fell to 8% in the HP groups, no matter the number of days of adaptation. In contrast, Demigne et al. (10) reported a net glucose release in rats fed a 50% protein diet. Despite a marked decrease in glycolytic enzyme expression as early as the first day of the HP diet (39), glucose oxidation progressively increased throughout HP adaptation (38), resulting in a negative CHO balance as reported in the present study. In contrast to amino acids, this adaptation occurs only in the fed state and not in the fasted state, in which glucose excess has been stored in tissues. Exploring whether insulin sensitivity varies at the same time would be an interesting question because it can control glycolytic enzyme activity (37) and glucose oxidation fluxes. The beneficial effect of HP diets on glucose tolerance has been reported in obese (4, 32) but not in lean rats (7, 18, 31). The effect of an HP diet on insulin sensitivity is also not elucidated since it has been shown to have no effect (4, 32) or to be detrimental (35).

We cannot exclude the possibility that amino acid carbon skeletons have been partially converted to ketone bodies. We could not, however, measure this process in the present study because it would have required complex procedures to measure ketone body production, using stable isotopes to measure ^{13}C in aceto-acetate and/or β-hydroxybutyrate. There are very few data in the literature addressing the impact of HP diets on ketones bodies, although it was recently reported that plasma β-hydroxybutyrate increased in humans (42) and in rats fed an HP diet (26). Interestingly, this study (26) reported that 2-ketoglutarate and fumarate were positively correlated to phenylalanine, methionine, and serine. Pyruvate and citrate also robustly distinguished the group fed a high-protein diet. These data also suggest that in addition to β-hydroxybutyrate, dietary amino acids could have been converted into ketoacids and other citrate cycle intermediates.

Finally, about 40% of the carbon skeletons produced from dietary amino acid deamination were not immediately oxidized. It is possible that in a 4-h period, deamination would be faster than complete oxidation of carbon skeletons. Accordingly, we made a similar observation in humans who were fed eggs in which proteins were intrinsically labeled with ^{13}C and ^{15}N. Four hours after the meal, almost half of the carbon skeletons were not oxidized, while after 8 h, deamination and oxidation were similar. Although this study was conducted under conditions of a normal protein meal (23 g), this supports the hypothesis that most nonoxidized carbon skeletons are temporarily retained in intermediary metabolic pools until oxidation. Under which form and in which tissues (intestine, liver, muscle) these nonoxidized carbon skeletons are retained remains an unanswered question.

Slowing of gastric emptying can also be suspected as a process involved in the adaptation to HP diets (23), but this point has seldom been addressed. To answer this question, we studied an additional group of rats to measure the amount of protein remaining in the stomach 2 and 4 h after the meal. Our results suggest a slowing of gastric emptying culminating after 3 days of adaptation. This supports the hypothesis of a buffering role of gastric emptying, probably to limit the massive flux of amino acid to the liver, while the deamination capacities are not maximized. Although not very pronounced, this observation is concordant with earlier observations that indicated delayed gastric emptying during the course of metabolism adaptation to a protein excess (23). Gut hormones, namely GIP, CCK, and glucagon-like peptide-1, could contribute to a modulation of gastric emptying, as an HP intake has been shown to stimulate their secretion (16). However, the effect of an HP diet on these hormones has been controversial in rats (31) and in humans (20). Additionally, it has also been shown that the feeding pattern and behavior sequence were markedly altered during the first day of the HP diet but returned to normal profiles as early as the second day (3).

Altogether, these results suggest that ingestive behavior is the earliest mechanism that takes place to cope with amino acid excess, followed by a modulation of gastric emptying during the first 3 days, and then by deamination enzymes that are optimally efficient between 3 and 6 days. However, this hypothesis requires some confirmation using sensitive and multiscale methods, including neurophysiology, imunohistochemistry, metabolism, and gene knockdown.

Perspectives and Significance

This study outlines the time course adaptation of amino acid handling through catabolic processes when feeding animals an HP diet. We showed that during adaptation to a drastic increase of protein in the diet, pathways of deamination are immediately more effective than those of oxidation. In addition, they are progressively upregulated, while we observed no adaptation of the pathways coping with oxidation.

We have previously shown that in rats fed ad libitum an HP diet, energy intake decreases in the short and the long term, and fat mass gain is reduced (3, 18). We propose that the postprandial accumulation of carbon skeletons acts as satiety signal, through activation of cellular energy sensors, such as AMPK, which is downregulated under an HP diet in the liver (9) and the hypothalamus (34). Moreover, as carbon skeletons from dietary amino acids are not used for glycogen or fat storage,

this may explain why the isoenergetic substitution of CHO by amino acids limits adiposity.

Further studies could be proposed to elucidate in which metabolic pools and in which organs carbon skeletons produced by AA deamination are preferentially retained until oxidation. Ketoacids and ketone bodies are good candidates, and the use of specific tracers could help to solve this question. Additionally, the selective deletion of the AMPK gene in the liver, muscle, and hypothalamus could enable a response as to whether the postprandial accumulation of carbon skeletons is involved in the satiating and metabolic effect of HP diets.

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Current affiliation for M. Stepien: Nutrition and Metabolism Section, International Agency for Research on Cancer (IARC-WHO), 69008 Lyon, France.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.S., D.A.-M., D.T., and C.G. conception and design of research; M.S., D.A.-M., P.C.E., N.K., and C.G. performed experiments; M.S., D.A.-M., P.C.E., N.K., and C.G. analyzed data; M.S., D.A.-M., P.C.E., and C.G. interpreted results of experiments; M.S. and C.G. prepared figures; M.S. and C.G. drafted manuscript; M.S., D.A.-M., P.C.E., G.F., D.T., and C.G. approved final version of manuscript; D.A.-M., P.C.E., G.F., D.T., and C.G. edited and revised manuscript.

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