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Postprandial oxidative losses of dietary leucine depend on the time interval between consecutive meals: a model study with rats

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ABSTRACT. Postprandial oxidative losses of egg white-bound [1-^{13}C]-leucine were studied as ^{13}C recovery in the breath of rats in relation to different time intervals between two meals. Male Wistar rats (n = 48; 68.3 ± 5.9 g) divided into 4 groups (n = 12) were fed two meals a day (9:00 and 16:30; interval 7 h) of a 13.2% egg white-based diet for 30 min. After 14 days, 3 out of the 4 groups received the 2nd meal at shorter time intervals of 3, 1, and 0 h. Two [$^{13}CO_2$] breath tests (BT) were performed on days 19 and 40. The breath samples were analyzed for ^{13}C Atom % (At %) enrichment by IRMS, and the results were expressed as the rate of ^{13}C At % excess and cumulative recovery (% of dose). The 7 h interval group showed higher ^{13}C cumulative recovery after the 2nd meal during both the BT and after the 1st meal on the day 40 BT compared with the 3, 1, and 0 h intervals. In groups with the 3, 1, and 0 h intervals, the cumulative recovery of ^{13}C after the 2nd meal was lower compared with the 1st meal due to interaction between meals, which probably caused dilution of the tracer in the larger volume of feed in the rat's stomach. On day 40, all cumulative recovery values were higher than on day 19. An important finding of this study is that despite the differences in postprandial Leu oxidation among interval groups, the weight gain of all rats was similar.

Introduction

Periodic eaters consume their total daily amount of feed in a restricted number of meals. This eating pattern should allow for storing nutrients during the postprandial phase (PP) to meet requirements between meals during the postabsorptive phase (PA) (Schreurs et al., 1997). The processes of protein loss during fasting and protein gain after feeding are called 'diurnal protein cycling' (Millward, 1995). After digestion and absorption, amino acids (AAs) from dietary proteins enter the free amino acids pool of the body and mix with endogenous amino acids. The metabolic utilization of dietary AAs may range from incorporation into body proteins to excretion as metabolic end products as CO_2, H_2O, urea (mammals) or uric acid (birds), and these processes might occur independently and concomitantly in

the body. The body pool of free AAs is kept small and constant relative to the amounts absorbed from dietary protein intake and the amount involved in body protein turnover. Absorbed dietary AAs, not used for protein synthesis, are channeled into energy metabolism (Schreurs et al., 1997). Postprandial storage of dietary amino acids can be impaired when postprandial oxidation of dietary amino acids is favored by, e.g., an excessively high appearance rate in the blood (Bos et al., 2003). Net protein synthesis can be influenced by endogenous factors (e.g., age, physiological condition, gender) as well as by exogenous ones (e.g., protein and energy intake, protein quality, protein bound vs free amino acids; Metges et al., 2000; D'Mello, 2003; Nolles et al., 2009).

An optimal nutritional strategy should channel dietary AAs into protein synthesis to minimize their PP oxidative losses. Feeding frequency and meal size are important nutritional factors that can influence the protein metabolism of the body. Studies on AA utilization in rats and fish suggest that the same marginal daily amount of food served as small and more frequent meals might improve protein utilization compared with less frequent larger meals (Schiffelers et al., 1996; Bujko et al, 1997, 2004; Borne et al., 2006; Ganzon-Naret, 2013). In the case of lower postprandial oxidation, more AAs remain available in the body until the PA phase to support growth, maintenance processes, or other functions, e.g., immune response (Bujko et al., 2004). On the other hand, increasing the number of meals from 2 to 4 and maintaining a constant feed dose (protein) for 25 days in growing rat females did not result in any significant impact on dietary protein utilization as measured by excretion of nitrogen and body weight gain (Myszkowska-Ryciak et al., 2006). In humans El-Khoury et al. (1995) reported even better 24-h leucine balance with three discrete meals compared with multiple small repeated meals. Similarly, Ohkawara et al. (2013) observed that increasing meal frequency from three to six meals per day has no significant effect on 24-h fat oxidation, but may increase hunger and the desire to eat.

It is hypothesized that the oxidative loss of dietary AAs during the PP phase of consecutive meals is modulated by the time interval between them. Due to metabolic adaptation, the interactions between consecutive meals might be changed after a period of conditioning on the experimental feeding schedule.

The aim of this study was to investigate the influence of different time intervals between two consecutive equal meals on PP oxidative losses of label from egg white-bound [1-^{13}C]-leucine present in the experimental meal by use of a [$^{13}CO_2$] breath test technique. A [$^{13}CO_2$] breath test is a simple and non-invasive method widely used in diagnostic applications as well as in studies on metabolic utilization of amino acids during the PP and/or PA phase of a meal (Raguso et al., 1999; Klein, 2001; Bujko et al., 2007). Body weight gain of the animals was monitored to reflect long-term protein utilization. Breath test (BT) results (^{13}C At % excess in expired CO_2 and total cumulative recovery of ^{13}C (% of dose/5 h) were compared at days 19 and 40 to study possible metabolic adaptation to the experimental feeding strategy. The protocol of the experiment was approved by the Ethics Committee of Wageningen University, Wageningen (the Netherlands).

Material and methods

Animals

Male WU-Wistar rats (n = 48, 3–4 weeks; Center for Small Laboratory Animals, Wageningen University, Wageningen, the Netherlands) were randomly divided into 4 experimental groups (n = 12) and caged individually at 22°C and 70% humidity with 16 h of artificial light and 8 h of red light (9:00–17:00). Drinking water was available *ad libitum* during the whole experiment.

Body weight gain

Body weight of individual animals was determined daily between 8:30 and 9:00 in the morning prior to feeding.

Feeding strategy

All animals were fed two equal meals a day for 30 min at 9:00 and 16:30 with a time interval of 7 h for 14 days. Total feed intake was monitored daily; the amount of feed for all rats for the next day was adjusted to the least eating rat. As a consequence all rats were served the same amount of feed every day.

After 2 weeks (on day 15) the time interval between meals was decreased for 3 groups to 3, 1, or 0 h, and for one group the interval remained at 7 h. The 7 h interval group was considered to follow the natural eating pattern of rats, whereas the 0 h interval group mimicked the consumption of one large meal daily. The two other time intervals were chosen to examine the utilization of AAs when the meal pattern entailed frequent or somewhat less frequent meals. Starting from day 18, the amount of feed was established at 11 g per day (according to the least eating rat) and maintained up to day 40. The meals during breath test (BT) measurements were reduced by 10% to the amount of 5 g each to eliminate possible refusals and to make the results comparable.

Table 1. Composition of experimental diets

Item	Diet, g · 1000 g^{-1}
Feed ingredients	
dextrose	194.5
wheat starch	475.0
egg white powder	132.0
soya oil	50.0
cellulose	75.0
vitamin premix	10.0
mineral premix	12.0
$CaCO_3$	19.0
$NaH_2PO_4 \cdot 2H_2O$	6.5
$KHCO_3$	10
KH_2PO_4	14
MgO	1.5
KCl	0.5
Analysed values	
crude protein	128.0
crude fat	50.0
crude fibre	73.0
alanine	7.6
arginine	7.1
asparagine	14.3
glutamine	15.9
glycine	4.2
histidine	3.6
isoleucine	7.3
leucine	10.7
lysine	7.6
methionine	4.5
methionine + cysteine	6.9
phenylalanine + tyrosine	12.8
proline	5.2
serine	9.7
threonine	6.2
tryptophan	2.4
valine	10.2

vitamin mix 10.1 g · kg^{-1}; mineral mix 12.1 g · kg^{-1}

During both the preliminary and experimental periods, the rats received the feed in pellet form. Four days prior to breath tests and during both BT measurements, the pellets were powdered and mixed with water to mash form.

Diet

Starting from day 1 all animals received a diet based on egg white protein (13.2%; 15.2 kJ ME· g^{-1}) in line with the requirements of rats (NRC, 1995). The diet composition is shown in Table 1. In the breath test meal (BTM), part of the protein was replaced by egg white with bound [1-^{13}C]-leucine (about 1.48 At % excess) produced according to the method by Evenepoel et al. (1997). During BTM the rats received 5 g of feed containing 0.66 g of protein (47 mg of non-labelled leucine and 6 mg of [1-^{13}C]-leucine).

[$^{13}CO_2$] breath test

[$^{13}CO_2$] breath tests were performed on days 19 and 40 of the experiment to monitor dietary AA utilization. For BT measurements half of the animals from each experimental group (n = 6) were given the tracer as a part of the 1st meal at 9:00. The other half of the animals received the label as a part of the 2nd meal, served after the appropriate time interval. The same animals were used on days 19 and 40. During the BT measurements the animals were individually kept in air-tight, macrolon cages (20 cm × 16 cm × 14 cm) bedded with sawdust and with access to water *ad libitum*. Every 30 min an air sample from the cage was taken with a 50 ml syringe to analyze the ^{13}C At % excess content. Air sampling started 30 min prior to the BTM to get a blank value for the natural ^{13}C enrichment of expired air. After each air sampling the rat was transferred to another cage with fresh air to start the next 30 min. Depending on the feeding schedule, the meals were presented in the cage at the appropriate time. In total, 11 breath samples were collected during 5.5 h of measurements for each animal.

The collected air samples were analyzed by a isotope ratio mass spectrometer (Finnigan MAT, Delta C, Bremen, Germany) for ^{13}C-enrichment at the WIAS IRMS Laboratory with an accuracy of ± 0.0005%.

The ^{13}C At % excess values (formula below) were plotted vs time.

At % Excess ^{13}C % = At % ^{13}C (sample n) – At % ^{13}C (sample 0).

The values for total cumulative recovery of label (% of dose recovered in 5 h) were obtained by summation of the amounts of label recovered in the sampling periods, and account being taken for CO_2 production during the sampling periods.

Expiration rate (% dose/h) = [(^{13}C At % excess × C expired/min × 60 min) / dose ^{13}C] × 100% ,

Cumulative recovery (% dose) = [sum of ^{13}C recovery in each sampling period / dose] × 100%.

CO_2 production

A value for CO_2 production is required to calculate the absolute ^{13}C recovery in expired air. In the present study, CO_2 production was measured for 8 rats (4 on day 19 BT, 4 on day 40 BT) from each time interval group by means of indirect calorimetry under conditions identical to the [$^{13}CO_2$] breath test. The normal feeding procedure was followed and measurements for

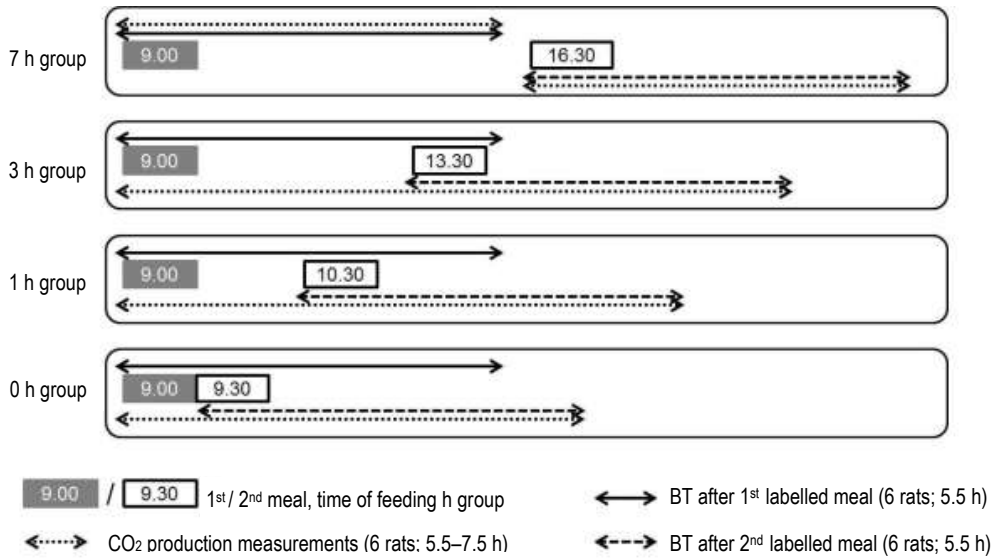

Figure 1. Scheme of the breath test/CO_2 measurements procedure. In each interval group half of rats received [1–^{13}C]-leucine in 1st meal (2nd meal not labelled) and the other half in 2nd meal (1st meal not labelled)

individual rats lasted for at least 5 h after the breath test meal. The cages were continuously ventilated with fresh air (about 0.5 l · min^{-1}). The outgoing air was dried over $CaCl_2$ and the CO_2 content of the air flow was measured using a continuous flow CO_2 analyzer (Uras-3G, Hartmann & Braun AG, Germany). CO_2 production was calculated as the average value for 30 min for individual rats. The values for CO_2 production (ml · min^{-1}) were corrected for standard conditions (0°C, 760 mm Hg, dry air).

The scheme of experimental procedure: labelled meals, breath tests, and CO_2 measurements is presented in Figure 1.

Statistical analysis

ANOVA/MANOVA variance analysis and the post-hoc LSD test (Statistica 10.0 StatSoft, USA for Windows) were used to determine differences, which were considered statistically significant at $P < 0.05$. Separate models were used to test: 1. the effect of different time interval on leucine utilization, 2. the effect of adaptation on leucine utilization, 3. the effects of interval and adaptation on leucine utilization.

Results

Body weight growth rate

Table 2 presents the rats' average weight at the beginning of the experiment and on day 19 and 40. There were no significant differences in initial body weight or in weight gain during the experimental period among the different interval groups.

Table 2. Average weight (g, mean ± SD) of rats at the beginning of experiment and during breath test measurements

Time interval groups, h	Day 1	Day 19 BT	Day 40 BT
7	67.0 ± 3.9	133.4 ± 4.2	180.8 ± 2.4
3	69.0 ± 8.3	134.3 ± 5.7	175.3 ± 6.3
1	69.0 ± 5.6	136.5 ± 8.2	180.1 ± 8.2
0	68.0 ± 6.0	129.2 ± 10.1	177.8 ± 6.6
average	68.3 ± 5.9	133.4 ± 7.9	178.5 ± 6.6

BT – breath test

CO_2 production

CO_2 production under the conditions of day 19 BT and day 40 BT (Figure 2) is presented as an average value measured in 16 animals. Carbon dioxide production decreased slightly after 17:00 in the group with the 7 h interval after the 2nd BTM due to the change in the light period (less active). The CO_2 production measured on day 40, on larger animals (+ 33%), was higher compared with day 19. However, the results showed no significant differences in either time or among different time-interval groups. Therefore, the mean values of CO_2 production for all animals (0.15 l CO_2/30 min for day 19 BT and 0.19 l CO_2/30 min for day 40 BT) were used for BT calculation.

[$^{13}CO_2$] breath test

The ^{13}C recovery of orally ingested egg white-bound [1-^{13}C]-leucine as part of the BTM was monitored for 5 h after the meal as [$^{13}CO_2$] in the breath. The pattern of ^{13}C recovery during the BT is presented as ^{13}C At % excess plotted against time.

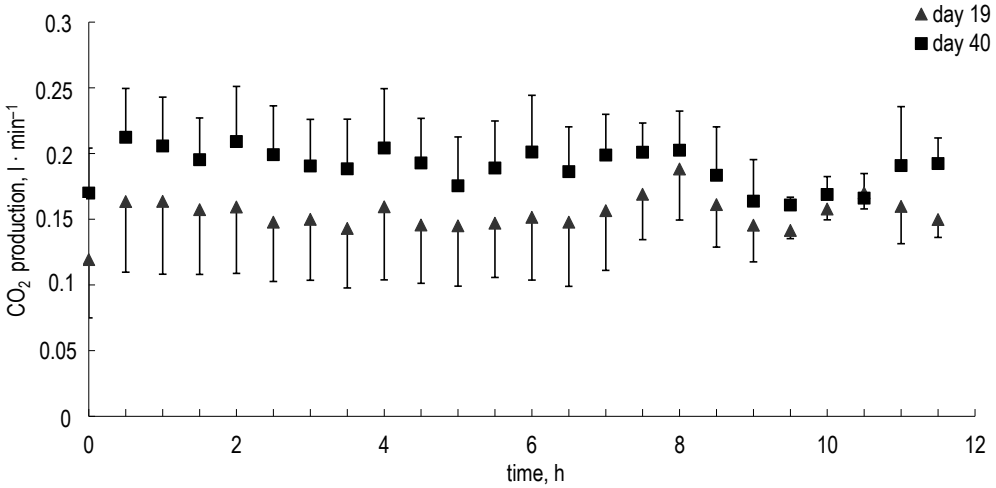

Figure 2. Average CO_2 production (mean ± SD) measured during day 19 and day 40 under breath test condition

The 1st BT was performed on day 19 of the experiment, 4 days after introducing the experimental time intervals between meals. The results (Figure 3) showed that in all cases, ^{13}C enrichment increased above the background level within 30 min. In no case did the value of ^{13}C enrichment return to the background level after 5 h of measurements, and it usually remained between 0.004% (0, 1 h intervals) and 0.009% (7 h interval). In the group with the 7 h interval, a similar pattern of ^{13}C At % excess was observed for the 1st and 2nd BTM. When the interval between meals was reduced (3, 1, and 0 h), the pattern of ^{13}C enrichment from the 1st and 2nd BTM changed reflecting the interactions between consecutive meals on PP leucine utilization.

The ^{13}C At% excess after the 1st BTM in the group with the 7 h interval increased rapidly to a value of 0.007% after 2 h. Thereafter the rate of increase slowed, and after 5 h a value of 0.009% was reached. The curve from the 2nd BTM was similar and reached a maximal value of 0.008% after 5 h. When the 2nd meal was given after a shorter time interval, the ^{13}C excess of both meals was affected. With an interval of 3 h, the response to the 1st BTM reached a level of 0.009% within 2 h and remained practically constant thereafter. The ^{13}C enrichment curve for the 2nd BTM reached a plateau of ca 0.005% after 3.5 h. When the interval was reduced to 1 h, the label recovery from the 1st BTM increased in the first 4 h to 0.009% and then started to decline.

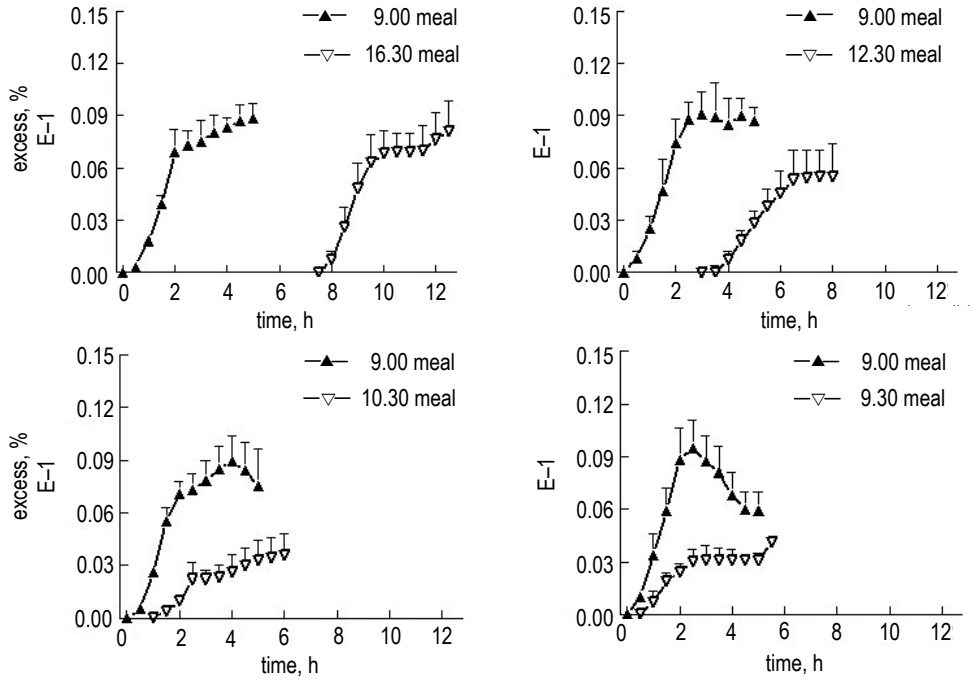

Figure 3. Rate of ^{13}C enrichment in breath (At % excess ± SD) derived from egg white bound [1-^{13}C]-leucine present in 1st or 2nd meal in groups fed with time intervals of 7, 3, 1 and 0 h during day 19 breath test

The curve of the ^{13}C excess from the 2nd BTM showed a biphasic increase to a value of 0.004% after 5 h. The curve of ^{13}C enrichment from the 1st BTM in the group with 0 h interval dramatically increased to a peak value of 0.01% within 2.5 h and then started to decline reaching 0.006% after 5 h. When the label was introduced in the 2nd meal, the curve of ^{13}C excess reached a plateau of about 0.003% after 2 h.

Results of the day 40 BT are presented in Figure 4. In general, the patterns of the curves of ^{13}C excess were similar to those observed on day 19, especially during the first 3 h of measurements. However, all values were slightly higher compared with the day 19 BT. During the last 2 h of BT measurements, all values of ^{13}C enrichment tended to increase compared with day 19 BT.

The results of the total cumulative recovery of ^{13}C from labelled leucine are presented in Figure 5.

Application of the label into the 1st vs 2nd meal significantly influenced the cumulative recovery

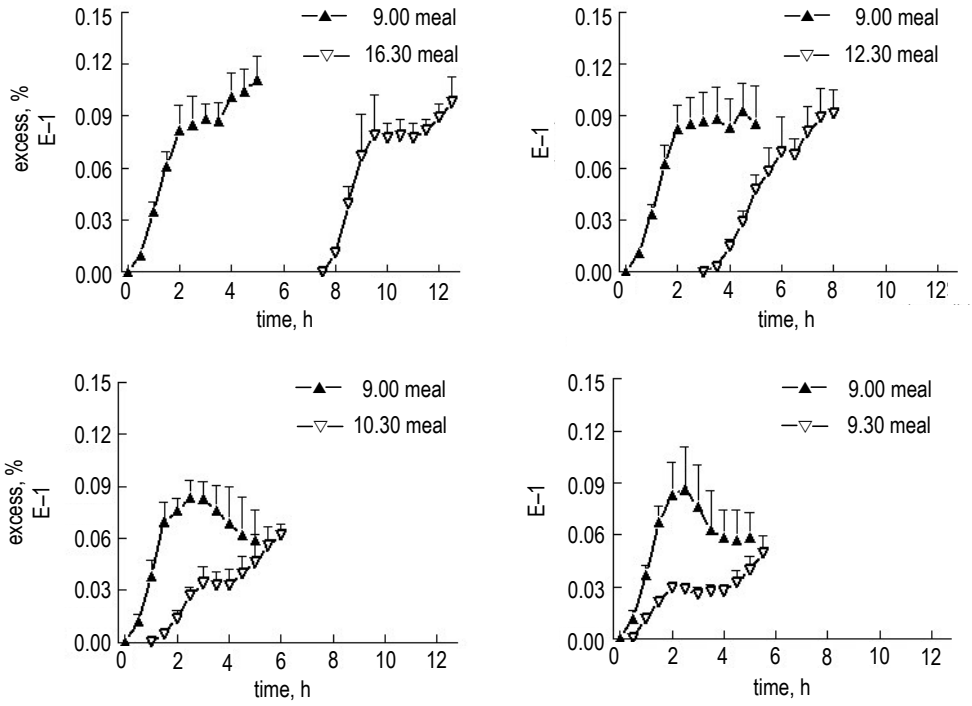

Figure 4. Rate of ^{13}C enrichment in breath (At % excess ± SD) derived from egg white bound [1-^{13}C]-leucine present in 1st or 2nd meal in groups fed with time intervals of 7, 3, 1 and 0 h during day 40 breath test

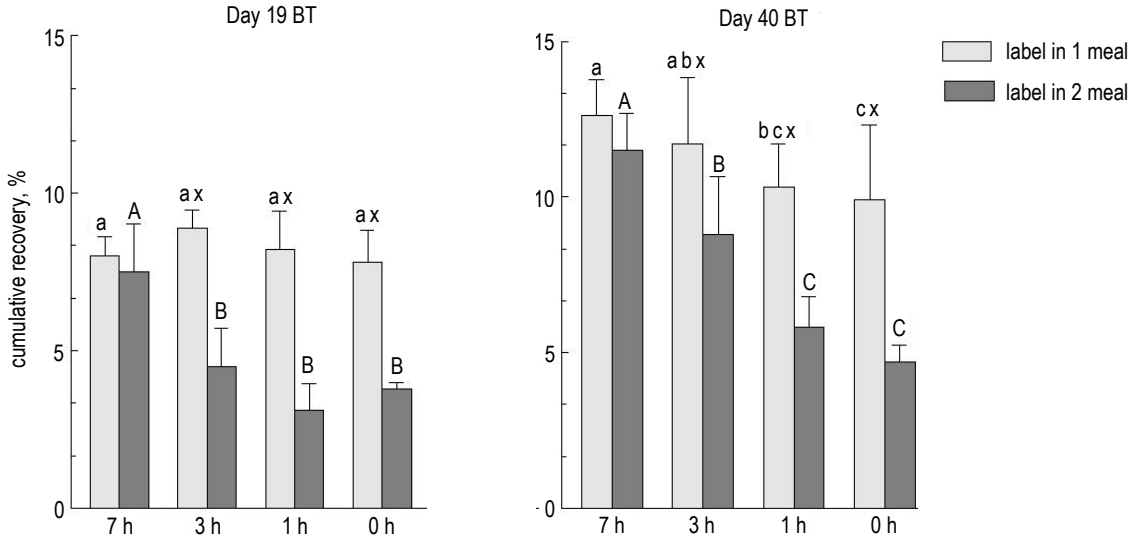

Figure 5. The total ^{13}C cumulative recovery of label introduced in 1st or 2nd meal after 5 h of breath test measurements (BT) in groups fed with different time interval (7, 3, 1 and 0 h) between meals (means ± SD). x – statistically significant difference between 1st breath test meal (BTM) and 2nd BTM within groups; a, b, c – statistically significant differences between 1st BTM in all intervals groups; A, B, C – statistically significant differences between 2nd BTM in all intervals groups

of ^{13}C, which was significantly higher for the 1st labelled meal in all groups on both breath tests except for the 7 h interval group. On day 19 there were no differences between the total ^{13}C recovery after the 1st BTM for all interval groups, whereas on day 40 differences were found. A significantly lower ^{13}C cumulative recovery was observed in groups with 0 h and 1 h intervals compared with the 7 h interval, but there was no difference between the 3 h and 7 h interval groups. In case of the 2nd BTM, the total cumulative recovery of ^{13}C was significantly highest in the 7 h interval group in both BTs and, additionally, on day 40 the 3 h interval group differed from the others.

Discussion

The aim of the present study was to test the hypothesis that oxidative losses of dietary [1-^{13}C]-leucine during the postprandial phase are modulated by the time interval between meals. In addition we examined if interactions between consecutive meals changed after 3 weeks of conditioning on a specific feeding strategy.

Since the first step of leucine catabolism is irreversible decarboxylation (Newby and Price, 1998), the amount of ^{13}C in the breath, measured after a [1-^{13}C]-leucine containing meal, reflects irreversible loss of dietary leucine. In other words only non-oxidized leucine remains available for metabolic processes of the body throughout the PA phase. As a consequence, lower total cumulative recovery of label during the PP phase is supposed to reflect a better nutritional situation with respect to utilization of dietary AAs. In humans a rapid, transient response of [$^{13}CO_2$] in the breath is observed after ingestion of [^{13}C]-labelled egg white. The maximal [^{13}C] enrichment is reached after about 120–145 min and then decreases nearly to the background level within 300 min (Ghoos et al., 1998; Geboes et al., 2004). The curve is characterized by an ascending part (charging of the metabolic pool) leading to a peak value for maximal excretion of label and a descending part (clearance of metabolic pool; Schreurs and Krawielitzki, 2003). A [$^{13}CO_2$] expiration curve represents the overall result of the flow of orally ingested label through all physiological and metabolic processes (gastric emptying, digestion, absorption, and metabolism). Results of human breath test lead to the conclusion that proteins ingested with a meal are almost immediately released by the stomach to the intestine for digestion and absorption. Therefore, bigger meals are expected to cause a higher appearance rate of amino acids in the blood within a short time (bolus application). As a consequence, higher oxidative losses of dietary AAs could be expected in the PP phase. In such a case the benefit of more frequent smaller meals compared with bigger meals consumed less often is possible and reported in many studies. In animals fed marginal amounts of protein more frequently, an improvement of dietary protein utilization was reflected in better weight gain (Schiffelers et al., 1996), better protein status reflected by AA oxidation, and even better immune response (Heatley, 1995; Bujko et al., 2004; Myszkowska-Ryciak et al., 2006).

As far as the ^{13}C appearance rate in the breath is proportional to meal size without restriction by gastric emptying, small meals consumed with too short time intervals might cause the same or even more negative effects on protein utilization. If a consecutive meal is eaten too early, when oxidation of dietary AAs from the first meal is maximal or on a relatively high level, then the overall oxidative loss is supposed to be higher. Thus, in our model study on rats we expected that with the shorter interval between equal consecutive meals, the cumulative recovery of label would be higher compared with the situation when two meals were more independent.

Our results show that despite meal ingestion taking a maximum of 30 min, the metabolic response, monitored by expiration rate of [$^{13}CO_2$] derived from egg white-bound [1-^{13}C]-leucine present in the meal, was not accomplished within 5 h as in humans. This suggests that the experimental meal cannot be handled as a 'single' meal (bolus) in rats due to prolonged amino acids oxidation. This might be explained in two ways. First, the handling of dietary amino acids on the metabolic level is not bolus-like because of a slow rate of gastric empting and/or digestion and absorption. Alternatively, rapid metabolic availability of amino acids is not followed by rapid and complete metabolic handling because of restricted metabolic capacity. It is remarkable that in our study in rats, one bolus-like meal created a [$^{13}CO_2$] response similar to the human response to repeated small boluses (nibbling) or slow protein feeding mimicking a constant feeding pattern (Boirie et al., 1997; Dangin et al., 2001).

It was also observed that responses to meals were independent with a 7 h interval, as the cumulative values as well as ^{13}C enrichment curves of both BTMs did not differ. A shorter time interval (3, 1, 0 h) between the meals influenced the ^{13}C enrichment of expired CO_2 after the 1st meal. The changes occurred during the last 3 h of BT measurements, whereas the patterns of ^{13}C At % excess during first

120 min were similar for all interval groups. This suggests that a substantial part of the ingested meal is directly transferred to the intestine for digestion and absorption. The rest of the meal remains in the stomach and can be mixed with a consecutive meal depending on the time interval. As a consequence, only part of the first meal is mixed with the 2nd meal in the stomach. Thus, the decrease in label recovery might be explained by dilution of tracer from the 1st meal by the non-labelled leucine in the 2nd meal. This effect is greater with a shorter interval between meals.

The low postprandial ^{13}C cumulative recovery after the 2nd meal (interval 3, 1 and 0 h) might be explained in a similar way by a 'queue' effect in the stomach. The 2nd meal with [1-^{13}C]-leucine was ingested while part of the protein from the 1st meal was still present in the stomach. These effects are also more pronounced with shorter time intervals between consecutive meals.

All values of ^{13}C cumulative recovery from labelled leucine were significantly higher (except the 2nd meal in 0 h interval) on day 40 than on day 19 of the experiment. The day 40 breath test measurements were performed on bigger animals (about + 33%) than on day 19. Thus, the same amount of feed as on day 19 was ingested to an increased stomach storage capacity, where mixing of consecutive meals was improved. Also the gastric empting rate may have been increased as well as intestine capacity might have been improved. As a consequence, a greater amount of tracer was available for metabolism in a shorter time. Furthermore, larger animals with a greater protein mass and lower growth rate have higher unavoidable oxidative maintenance losses of amino acids compared with smaller animals with higher growth rates.

ANOVA results showed an effect of adaptation in different interval groups on day 40. The feeding conditions (amount of feed and label) were exactly the same during both BT measurements and the rate of growth did not differ among interval groups. Therefore, the lower cumulative recovery of ^{13}C in the 0 and 1 h interval groups reflected an improvement in utilization of dietary leucine compared with the 7 h interval. In this case, some gastrointestinal functions could have adapted to a larger amount of protein being ingested in a short time (0, 1 h intervals) by decreasing the inflow of amino acids to the metabolic pool. Shi et al. (1997) reported that gastric emptying of a protein meal can be modified by previous dietary protein intake during at least 14 days. On the other hand, adaptation to altered feeding by decreasing the oxidation rate through the physiological mechanism of diurnal protein cycling might have occurred. This mechanism may adjust the rate of amino acid utilization after a meal to the composition and amount of diet (Millward, 1995).

Our results show that time intervals between meals modulate oxidative loss of dietary leucine. Because a single meal in rats is not treated like a bolus in the metabolism and because the response is not accomplished within 5 h, the present model study cannot provide a clear conclusion about the overall leucine utilization. However, it can at least be assumed that different time intervals between consecutive meals did not influence long-term protein utilization as measured by growth of the animals. Our observations support the idea of the important role of gastric empting rate on the regulation of amino acid utilization in the rat. The deceleration of gastric empting in response to the saturation of catabolic capacities by a meal high in protein was previously reported by Morens et al. (2000). Türker and Yildirim (2011) showed that because of slower digestion, a six-meal feeding strategy was more efficient in terms of weight gain compared with 2, 3, or 4 meals. The speed of protein digestion and amino acid absorption from the gut also plays an important role for whole-body protein anabolism after a single meal in humans. Boirie et al. (1997) showed that slowly absorbed casein promotes postprandial protein deposition by inhibition of protein breakdown without excessive increase in amino acid concentrations, and on the other hand, a fast dietary protein (whey) stimulates protein synthesis, but also oxidation. It is clear that humans and rats react differently to meal size. In humans the reaction is more restricted by metabolic capacity, whereas in rats the reaction is more influenced by metabolic availability.

The important result of the study is the fact that regardless of the interval between meals and differences in leucine oxidation, weight gain, reflecting the protein status of the animal was the same.

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