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Thermochimica Acta 349 (2000) 53–59

thermochimica acta

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Breath test measurements in combination with indirect calorimetry for estimation of ^{13}C-leucine oxidation in mink (*Mustela vison*)

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Received 7 September 1999; received in revised form 27 October 1999; accepted 1 November 1999

Abstract

Gas exchange measurements by means of indirect calorimetry can be used to calculate quantitative substrate oxidation. The results represent average net oxidation values (substrate disappearance rate), but they cannot describe the dynamics of the oxidation processes. Breath test measurements with substrates labelled with ^{13}C provide an attractive tool to describe the dynamics of oxidation processes, and may in combination with indirect calorimetry refine estimation of substrate oxidation. The objective of our investigation was to estimate oxidation of 1-^{13}C labelled leucine in mink in response to feeding and fasting. Twelve 1-year-old male mink (*Mustela vison*) were measured in each five consecutive periods by means of indirect calorimetry and simultaneous breath test. In Periods 1, 3 and 5, each lasting 3 days, the animals were fed ad libitum and Periods 2 and 4 were fasting periods, each of 48 h. In Periods 1 and 5 all animals were fed a diet with a high quality fish meal (FISH; n=12), while in Period 3 half of the animals received the FISH diet (n=6) and the other half a diet with soy protein concentrate (SOY; n=6) as main protein source. An intraperitoneal injection of 1-^{13}C-leucine was given before measurements started and expired air was then sucked out of the respiration chamber and collected into breath bags at frequent intervals until 5.5 h after the start of measurements. The ratio of ^{13}C/^{12}C was measured by means of an IRIS infrared analyser and results are reported in terms of delta over baseline (DOB) values. There was no significant effect of dietary treatment group, but the interaction between treatment group and sampling time was significant (P=0.02), peak DOB values being recorded 70–135 min after injection in FISH animals, and 70–120 min in SOY animals. The effect of period was significant (P=0.03), values generally being lower during fasting, indicating a lower rate of leucine oxidation. It was concluded that the present results clearly demonstrate differences in rate of oxidation of leucine between fed and fasted animals.

Keywords: Mink; Calorimetry; ^{13}C-Leucine; Oxidation

1. Introduction

Quantitative oxidation of nutrients in the intact organism can be calculated based on gas exchange measurements by means of indirect calorimetry and

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0040-6031/00/$ – see front matter
PII: S0040-6031(99)00496-7

urinary excretion of nitrogen. This technique has been used for humans [1] and its validity has been shown for growing pigs [2,3] and later also for rats and mink [4]. It has, furthermore, given reasonable estimates of nutrient oxidation for ruminants as demonstrated in growing calves on differentiated diets [5]. This type of calculation gives the net oxidation rate (substrate disappearance rate) of each nutrient over the total experimental period, which in the case of indirect calorimetry is often 24 h, but it cannot describe the dynamics of the oxidation processes. There are some inborn limitations with the method. For instance, the glycogen stores of the body must remain constant over the experimental period. Calculation of protein oxidation presents a problem since it is calculated from the total amount of nitrogen excreted in urine (UN), and the results, therefore, mirror protein deamination rather than total oxidation. Furthermore, it is has been stated that results are only valid if the respiratory quotient (RQ) ranges between 0.7 and 1.0, and that erroneous results are achieved in situations with prevailing lipogenesis (RQ above 1.0) or gluconeogenesis (RQ below 0.7) [6], but Chwalibog et al. [3] have demonstrated that this method can be used also when RQ exceeds 1.0.

Breath test measurements comprise collection of expired air from the experimental subject. By using a substrate labelled with ^{13}C or ^{14}C the ratio between the ^{13}C or ^{14}C and ^{12}C in CO_2 can be used as a measure of the rate of oxidation of the substrate. In the case of protein oxidation leucine is a commonly used substrate and Schreurs et al. [7] have demonstrated that it is a suitable substrate for describing the dynamics of the oxidation process. When using 1-C labelled substrates the breath test will monitor the total decarboxylation of the substrate, whereas when uniformly labelled substrates are used the total oxidation will be monitored [7,8]. Simple breath test measurement is more qualitative than quantitative, but it can, if air flow and CO_2-concentration are measured, be used as a quantitative method. Therefore, the combination of indirect calorimetry and breath test measurements seems to offer an attractive tool for studying both the dynamics and the quantitative results of oxidation processes in intact animals.

Nutritional factors not only have short-term effects on growth, body composition and body function, behaviour, welfare and health but also exert long-term effects on these body characteristics [9,10]. The possibility that nutrients consumed during the first weeks and months of life may have permanent effects on adult metabolism was recognised by McCane [11], Dietz et al. [12] and Lucas [10]. The metabolic basis of these long-term effects, a phenomenon called 'metabolic programming' [13], 'nutritional memory' or 'metabolic hysteresis', [9,14] is almost unknown. It is assumed that the interaction of nutrients and gene expression may form the basis for these programming effects [15,16]. For practical purposes the programming of permanent effects of the physiology and function of the organism during critical time periods of development (prenatal and postnatal) appears to be of major importance in preventive strategies for promoting and sustaining of performance and health. A better definition of the optimal supply of nutrients during critical early periods of life is thus expected to help the developing organism to utilise its genetic and metabolic potential with lasting positive effects for the future life.

The objective of this study was first to develop a technique for the combination of indirect calorimetry and breath test measurements for estimation of protein oxidation, and second, to attempt to develop an experimental model for the study of metabolic programming effects with use of mink (*Mustela vison*) as a model animal.

2. Material and methods

Twelve 9–10 months old male mink of the standard black colour type [17] and with a live weight of approximately 2.0 kg were used. The animals were transferred from our experimental farm to metabolism cages [18] in the laboratory one week before the first balance period. They were measured in three balance periods of 3 days each with two fasting periods of 2 days each between the balance periods. Six animals were fed a diet based on a high quality fish meal (LT, Tyborøn, Denmark) as a reference protein throughout all balance periods whereas the remaining six animals were fed a diet based on soy protein concentrate (Soycomil K, Loders Croklaan, Wormerveer, The Netherlands) as a test protein during balance Period 2. The diets were designed so as to be iso-nitrogenous and iso-energetic but with differences in the supply of

Table 1
Dietary composition in percent, calculated contents of digestible protein, fat, carbohydrate, lysine and methionine and metabolisable energy

	Protein source	
	FISH	SOY
Ingredients (g kg^{-1})		
Fish meal, LT	140	–
Soy protein concentrate	–	150
Whole chicken	100	110
Soy-bean oil	30	44
Potato mash powder	120	110
Maize starch	20	20
Vitamin/mineral mixture	2.5	2.5
Water	588	564
Calculated contents of digestible nutrients (g kg^{-1})[a]		
Protein	105	105
Fat	56	58
Carbohydrate	83	81
Calculated contents of digestible amino acids (g 16 g N^{-1})[a]		
Lysine	8.8	7.6
Methionine	3.1	1.4
Calculated content of metabolisable energy (kJ kg^{-1})[b]	5650	5685

[a] Calculated by use of feed tables and digestibility coefficients for the ingredients [19,20].

[b] Calculated from the contents of digestible protein, fat and carbohydrate and with use of metabolisable energy coefficients 18.4, 39.8 and 17.6 for protein, fat and carbohydrate, respectively [21].

essential amino acids (Table 1). The dietary protein content was chosen so as to be sufficient to cover the animals' protein requirement [21] and feed was supplied ad libitum. Access to drinking water was free at all times. The sequence of balance and fasting periods and source of protein supply to the respective animals are given in Table 2.

During each period a 22 h respiration experiment by means of indirect calorimetry in an open-air circulation system and a 5.5 h breath test measurement was performed on Day 2. Quantitative collection of faeces and urine (in 10 ml 5% H_2SO_4) was performed each day at 9.00 h. Animals were weighed at the start and end of each period. Feed, faeces and urine were analyzed for N by a micro Kjeldahl method in a Tecator-Kjeltec 1030 (Tecator AB, Höganäs, Sweden) system. Nitrogen balances were calculated as retained nitrogen (RN)=ingested nitrogen (IN)−faecal nitrogen (FN)−UN. Data for UN were corrected for incomplete recovery during collection according to Tauson et al. [22] who showed that even when using very careful collection routines, the loss is in the order of 20%. Heat production (HE) (not reported here) was calculated according to Brouwer [23]. Oxidation of protein (OXP), fat (OXF) and carbohydrate (OXCHO) (data not reported here) were calculated as described and validated for pigs by Chwalibog et al. [3].

Table 2
Sequence of balance and fasting periods and dietary treatments

Animals	Period				
	Balance 1	Fasting 1	Balance 2	Fasting 2	Balance 3
1–6	FISH	FAST	FISH	FISH	FISH
7–12	FISH	FAST	SOY	FAST	FISH

For breath test measurements 10 mg 1-^{13}C-leucine (99 at.%, Euriso-top, Saint Aubin Cedex, France) dissolved in sterile isotonic saline (9 g l^{-1}) was administered as a intraperitoneal injection and the animals were immediately put in the respiration chambers which were quickly closed. The first sample of expired air was taken 10 min after injection. During the first 2 h samples were drawn every 10 min, then for 1.5 h each 15 min and for the remaining sampling period every 20 min. A sample taken when the respiration experiment was ended after 22 h was used as zero sample (baseline value). Air was drawn from the chambers into breath bags with a volume of approximately 11 by means of a laboratory pump (Ecoline V280, Ismatec). The breath bags were filled in approximately 30 s. The ratio between ^{13}C and ^{12}C in expired CO_2 was measured by means of an IRIS infrared analyzing system (Wagner Analysentechnik, Bremen, Germany). Results are reported as delta over baseline values (DOB), per mil.

Statistical analyses of breath test measurements were performed according to the GLM-procedure in Statistical Analysis System [24] by use of the following model:

$$Y_{ijklm} = \mu + a_i + b_j + c_k + ab_{ij} + ac_{ik} + bc_{jk} + abc_{ijk} + d_l(ab)_{ij} + e_{ijklm}$$

where Y_{ijklm} is the Y_{ijklm}th observation, μ the general mean, a_i the fixed effect of treatment group (FISH/SOY), b_j the fixed effect of period (Balance 1–3 and

Fasting 1–2), c_k the fixed effect of sampling time; ab_{ij}, ac_{ik}, bc_{jk} and abc_{ijk} the interaction effects between treatment group and period, treatment group and time, period and time, and treatment group period and time, respectively, $d_l(ab)_{ij}$ random animal within treatment group and period and e_{jiklm} is the random error. Random animal within treatment group and period was used as an error term when testing effects of treatment group.

Similarly, the effects of animals being in the fed or fasted state, or fed only on the fish or combined fish and soy diets, respectively, were evaluated with a model comprising the fixed effects of state (fed or fasted) or dietary treatment (only FISH or FISH, SOY, FISH) and sampling time, the interaction effects between state or dietary treatment and time and random mink within state or dietary treatment. The latter term was used as error term when evaluating effects of state or dietary treatment. Results are reported as least squares means (ls-means) and pair-wise comparisons between ls-means were tested by a *t*-test with comparison-wise error rate 5%.

Nitrogen balance data were analysed by use of the MIXED-procedure in SAS [25] using group, period and sampling time as fixed effects.

3. Results

Breath test measurements showed that the appearance of labelled CO_2 in expired air increased steeply soon after injection. Already at the first sampling 10 min after injection, values were significantly above the baseline values. Peak values were attained 80 min after injection, and in the period 70–135 (FISH) and 70–120 (SOY) minutes after injection values were not significantly different from peak values, the interaction between group and sampling time being significant (P=0.02). From then on values started to decline and at the end of the sampling period DOB-values were low, but still significantly above baseline values. The effect of dietary treatment group was non-significant (Fig. 1). This was also the case during balance Period 2 when the animals were fed different diets (Fig. 2). There were significant period effects (P=0.03), DOB-values generally being lower in the fasting periods than during feeding. When data were evaluated regarding the effect of animals being in the fed or the fasted state, the oxidation curve had the same general appearance in fed and fasted animals. However, the oxidation rate was lower in fasted animals (P=0.04) as indicated by lower DOB-values from the peak and until the end of the sampling period (Fig. 3).

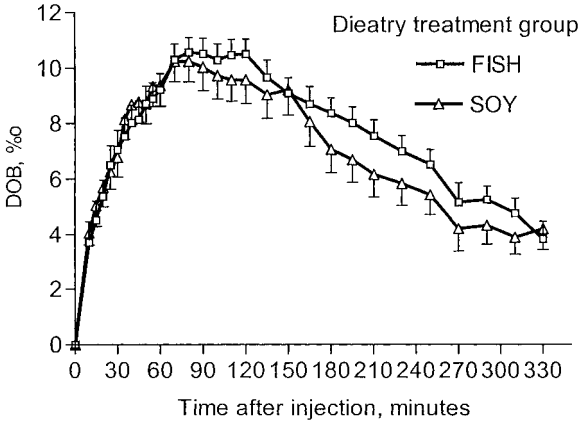

Fig. 1. Effect of dietary treatment group (FISH or SOY) on rate of 1-^{13}C-leucine decarboxylation by means of breath test measurements in adult male mink. Data represent ls-means and SEM over 3 balance and 2 fasting periods. Effect of dietary treatment group was non-significant.

Nitrogen balance data (Table 3), similarly, were independent of dietary treatment group but there were highly significant period effects (P<0.001). During balance period 1 animals appeared

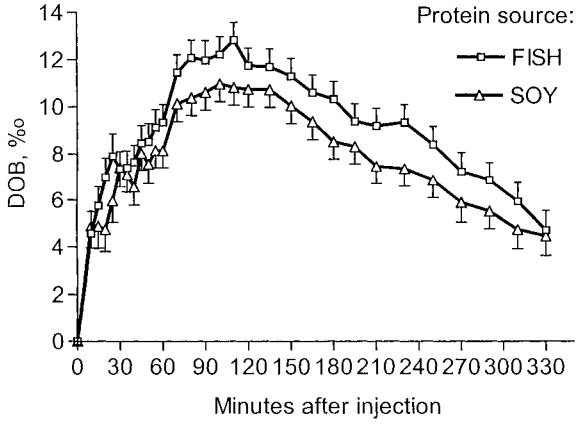

Fig. 2. Pattern of decarboxylation of 1-^{13}C-leucine by means of breath test measurements in adult male mink fed a diet based on high quality fish meal (FISH) or soy protein concentrate (SOY). Effect of protein source was non-significant.

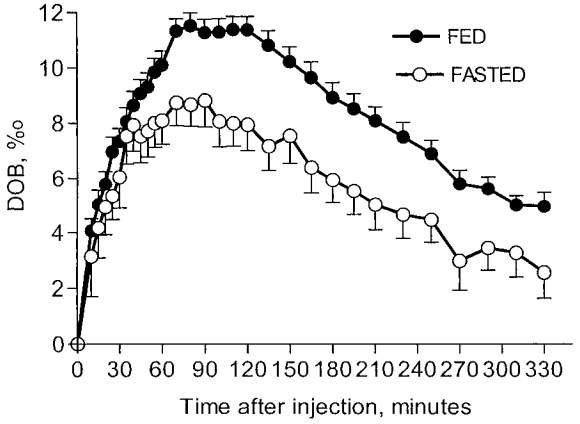

Fig. 3. Pattern of decarboxylation of 1-^{13}C-leucine by means of breath test measurements in adult male mink in the fed or fasted state. Differences between fed and fasted animals were significant (P=0.04).

to be in a slightly positive N balance, which was turned into clearly negative values during fasting periods 1 and 2. In balance periods 2 and 3 N balances were again positive, and for animals in the FISH group significantly higher than during balance period 1 (P<0.001 and P=0.003, respectively). In FISH but not in SOY animals RN made up a significantly higher proportion of DN in balance periods 2 and 3 than in balance period 1, indicating a better utilization of dietary protein during refeeding. Urinary N made up 82 (fed, fish diet), 86% (fed, soy diet) and 87% (fasted animals) of the total daily N excretion. Calculated in terms of OXP oxidation of protein contributed with 355 kJ (fed, fish diet), 320 kJ (fed, soy diet) and 125 kJ (fasted animals) to the total daily HE.

4. Discussion

The mink was chosen as a model animal because it can be fed diets which mimic human diets regarding proportion of ME provided by protein, fat and carbohydrate, respectively. Moreover, it is a small mammal, and therefore suitable for studies with stable isotopes which may be too expensive for studies in larger

Table 3
Nitrogen metabolism data: ingested nitrogen (IN), faecal nitrogen (FN), digested nitrogen (DN), urinary nitrogen (UN) and retained nitrogen (RN), g 24 h^{-1}, and RN/DN (%) of male mink fed a diet based on fish meal (FISH) in balance periods 1–3 or based on soy protein concentrate (SOY) during balance period 2

	IN		FN		DN		UN[a]		RN[a]		RN/DN	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Balance period 1												
FISH	3.73	0.264	0.62	0.056	3.10	0.238	2.86	0.273	0.24	0.262	4.8	7.14
SOY	4.24	0.286	0.63	0.060	3.61	0.259	3.10	0.292	0.55	0.289	12.7	7.75
Fasting period 1												
FISH	0		0.20	0.055	0		1.09	0.269	−1.34	0.264		
SOY	0		0.12	0.055	0		0.88	0.269	−1.14	0.264		
Balance period 2												
FISH	4.79	0.262	0.65	0.055	4.14	0.237	2.51	0.269	1.56	0.264	37.4	7.20
SOY	3.97	0.262	0.42	0.055	3.54	0.237	2.64	0.269	0.75	0.264	20.7	7.20
Fasting period 2												
FISH	0		0.13	0.056	0		0.93	0.273	−1.07	0.263		
SOY	0		0.21	0.056	0		1.13	0.273	−1.36	0.263		
Balance period 3												
FISH	5.13	0.260	0.72	0.055	4.43	0.236	3.17	0.266	1.30	0.262	29.4	7.14
SOY	5.14	0.260	0.75	0.055	4.41	0.236	3.56	0.266	0.88	0.262	20.1	7.14
P-value; effects of period[b]	<0.001		<0.001		<0.001		<0.001		<0.001		0.02	

[a] Corrected for incomplete (80%) recovery of UN according to Tauson et al. [22].
[b] Effects of dietary treatment group and interactions between period and treatment group non-significant.

species. In this experiment a moderate protein supply was given, but it was still sufficient to cover the animals' protein requirement, a fact that probably has influenced the results.

Breath test measurements showed that the response to the injection was rapid, results conforming with data from rats [7], and that values started to decline towards baseline values approximately after 2 h. The lack of significant effects of the dietary treatment probably can be explained by the fact that the diets were providing protein above the animals' requirement, and since the animals were almost mature it is not likely that prominent 'nutritional memory' effects could be documented. Junghans et al. [9] have previously shown differences in the pattern of protein oxidation, and persisting changes in the level of oxidation in young growing pigs fed diets based on casein or soy protein isolate after a change of the protein source. But, their animals were only supplied with about half the protein requirement, and the animals were in a less mature physiological state than the animals in our experiment. Likewise, Weijs et al. [26] have shown that dietary conditioning of rats prior to breath test measurements influenced the results in both growing and adult animals, but compared to our experiment their animals were conditioned to low or high protein diets for a considerably longer period before measurements.

The pattern of feed supply is also likely to influence the oxidative processes. Animals fluctuate between post-prandial periods of gain and post-absorptive periods of mobilisation of dietary proteins, a phenomenon termed diurnal protein cycling [27], and in the extreme case of a total fast, animals will in due time start to mobilise body protein stores. Hence, several small meals per day seems favourable regarding the animals' ability to economise with dietary protein compared with the same protein supply given in two large meals [8,28]. The mink usually consumes several small meals throughout the day, but when living in the wild and when no prey is available, it may experience protracted fasting periods. In this study the animals had food available during breath test studies in the feeding periods, and animals in the fed state had a significantly higher rate of leucine oxidation as compared with fasted animals. These results conform with unpublished data from Schreurs [29]. It is also consistent with findings of Schreurs et al. [7] and Weijs et al. [26] showing that animals with a low protein supply, or fed protein free diets, decarboxylate and oxidize amino acids at a lower rate than animals with a high protein supply. Also, from the nitrogen balances it was evident that deaminated protein contributed less energy during the fasting periods than during feeding, indicating a lower rate of oxidation. In agreement with previous studies [22,30], mink start to mobilise body protein stores during a 48 h fasting period. In the refeeding periods following fasting, the animals were in positive nitrogen balance, which at least for FISH animals was significantly higher than during the first balance period. Moreover, in FISH animals RN made up a significantly higher proportion of DN during the refeeding periods, indicating that the animals were repleting their body protein stores, and when doing so were able to utilize dietary protein more efficiently.

In conclusion, the present study has described a model for the combination of breath test and indirect calorimetry measurements. It was, furthermore, clearly shown that the rate of oxidation of 1-^{13}C-leucine differed between animals in the fed and the fasted state. The experimental model described seems, with some modifications, to be a promising starting point for studies of metabolic programming effects in intact animals.

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