^{13}C Enrichment of the CO_2 in Breast Milk and in the Breath Is Rapidly Modified by Changes in the ^{13}C Content of the Diet

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Key Words

^{13}C enrichment · Breast milk · Breath CO_2 · ^{13}C content of diet · C3 and C4 plants

Abstract

Background: C4 plants (e.g. corn and sugar cane) have greater ^{13}C enrichment than C3 plants (e.g. wheat and sugar beet).

Objective: To assess whether ^{13}C enrichment of CO_2 in the breath and breast milk of women on diets based on C3 and C4 foods changes from one diet to the other. **Materials and**

Methods: Six breast-feeding women were studied at 5–6 months postpartum. They ate a controlled C4 diet on days 1 and 2 followed by a C3 diet on days 3 and 4. Diet duplicates, breast milk on days 2 and 4 and hourly breath samples were collected over 4 days. ^{13}C enrichment was measured by isotope-ratio mass spectrometry. Values of $\delta^{13}\text{C}$ were calculated from the international PDBV standard ($\delta^{13}\text{C}_{\text{PDBV}}$). Differences between means were compared by paired t test or t test for repeated measurements. **Results:** $\delta^{13}\text{C}_{\text{PDBV}}$ values were significantly higher in the C4 diet than in the C3 diet composites ($p < 0.01$). In breath CO_2 , the $\delta^{13}\text{C}_{\text{PDBV}}$ value was greater on days 1 and 2 (range –15.4 to –13.2, respectively) and declined

on days 3 and 4 (range –20.0 to –21.8, respectively, $p < 0.01$). The lipid and milk serum fractions of breast milk had significantly higher $\delta^{13}\text{C}_{\text{PDBV}}$ on the C3 diet than on the C4 diet ($p < 0.01$). **Conclusions:** Subjects eating a C4 diet have a higher $\delta^{13}\text{C}_{\text{PDBV}}$ value in the breath and breast milk fractions, which diminish rapidly on a C3 diet. Further studies focusing on individual nutrients are warranted. © 2014 S. Karger AG, Basel

Introduction

Plants incorporate carbon atoms from atmospheric CO_2 into complex molecules by photosynthesis via the Calvin cycle (e.g. C3 plants like wheat, rice, nuts and potatoes) or the Hatch-Slack cycle (e.g. C4 plants like corn, sugar cane and some amaranthaceous plants) [1–4]. C4 plants preferentially incorporate ^{12}C , but C3 plants have a greater preference for ^{13}C . This results in the higher ^{13}C content of C4 plant products compared to C3-derived products, i.e. carbohydrates, lipids and, to a lesser extent, protein.

Animals of the same species that are fed diets with differing contents of C3 and C4 plants [5] have different ^{13}C enrichments in their expired CO_2 , serum and breast milk.

Some investigators reported differences in the background ^{13}C enrichment of expired CO_2 between European and American men during exercise. Such a difference was attributed to a higher content of C3 plants (i.e. potato and sugar beet) in the European diet. To the contrary, there is a higher content of C4 plants (i.e. corn and sugar cane) in the American diet [6]. Children drinking milk from cows that are fed C4 plants have greater $^{13}\text{CO}_2$ enrichment in their expired air than those drinking milk from cows fed with C3 plants [7, 8]. Several studies have used C4 plant products as tracers to study metabolic pathways [9–11]. In a study from our laboratory, we found a greater background ^{13}C enrichment in the fat tissue of lactating women eating a corn-based diet, compared to the fat of women eating a wheat-based diet [Villalpando, unpubl. data]. Based on the findings from that study, we hypothesized that their fat deposits must have greater natural ^{13}C enrichment. We propose devoiding the diet of C4 plant products from lactating women it could be possible to trace the flow of naturally ^{13}C -enriched nutrients mobilized from the circulation or body stores into expired air and breast milk.

The objective of this study was to establish whether, under controlled metabolic conditions, we could observe changes in the ^{13}C enrichment of CO_2 in the expired air and the fractions of breast milk samples from women on diets based on foods from C3 and C4 plants, sequentially.

Materials and Methods

Subjects

Six healthy women attending the local prenatal clinic of San Mateo Capulhuac, Mexico, who were predominantly breast-feeding their infants, were enrolled for the study at 5–6 months postpartum. The following inclusion criteria were met: they had had an uneventful pregnancy, they had delivered singletons weighing $>2,500$ g, they were between 18 and 34 years old, had a parity <4 , a height of between 145–154 cm, were free of any chronic disease and did not take any medication or drink alcohol regularly. The characteristics of the village have been described elsewhere [12]. In short, it is located in a mountainous area, 150 km northwest of Mexico City, at 2,800 m above sea level and has 5,500 inhabitants. They live on subsistence agriculture, with maize as their main staple. The habitual dietary intake of lactating women includes an energy intake of 48.2 ± 6.1 kcal/kg of body weight, and 0.8 ± 0.1 g of protein/kg. Carbohydrates provide about 75% and lipids about 17% of the energy intake. Cooking oil (mostly sesame seed, a C3 plant and sorghum, a C4 plant) is the main source of dietary lipids, eaten as a combination of 73 and 27%, on average [12].

The study protocol was reviewed and approved by the Ethics Committee of the Instituto Mexicano del Seguro Social. Written informed consent was obtained from all subjects after carefully explaining the nature, procedures and burdens of the study.

Methods

Subjects were admitted to the local health facility on the morning of day 1 for 5 days. Basal samples of breath and breast milk were obtained at 7 a.m. All women were encouraged to maintain their habitual daily activity pattern (i.e. cooking, cleaning the house, laundry, etc.) within the grounds of the health center.

Habitual Dietary Intake and Design of Experimental Diets

The usual dietary intake of the subjects was assessed during the week prior to the study by a combination of test-weighing for 2 days and 24-hour recall for 1 day in their households. Macronutrients and energy intakes were calculated by comparison with Mexican food composition tables [13]. Based on this information, we designed two controlled diets that resembled the nutrient composition and energy density of the habitual diets of these women. One was based on corn and cane sugar as staples, representing 70% of the energy intake. This diet had greater ^{13}C enrichment, and will be referred to from here on as the C4 diet. This was eaten by the subjects on the first 2 days of the study. The second controlled diet was based on wheat products, bee honey and vegetables belonging to the C3 family. This diet, referred to from here on as the C3 diet, had a lower ^{13}C enrichment, and was eaten by the subjects on days 3 and 4 of the study. Other vegetables classified as CAM (combining the metabolic characteristics of C3 and C4 plants), like a cactus eaten frequently at that locality, were avoided. Both controlled diets were isoenergetic and contained the same proportion of macronutrients; no changes were introduced in the composition of fat and protein. The sources of fat and proteins: milk, chicken meat, eggs and edible oil were not changed in either experimental diet. The women's individual energy intake was calculated according to their reported habitual energy intake, but if the reported intake was <40 kcal/kg $^{-1}$ /day $^{-1}$, the diet was adjusted to yield enough energy content. Meals were provided according to a fixed timetable, in accordance with the cultural norms of the community and the convenience for the study, i.e. breakfast at 7 a.m., lunch at 1 p.m. and supper at 7 p.m.

Composites of both types of diets were collected, homogenized, and frozen at -20°C until the analysis of total fat content by gravimetry, and an acid precipitation with trichloroacetic acid procedure for protein and the energy content was performed using an adiabatic calorimeter (Parr, Model 1266, Parr Instruments Co., Moline, Ill., USA) and ^{13}C enrichment by isotope-ratio mass spectrometry.

Milk Sample Collection and Fractionation

Milk samples were collected by emptying the left breast with an electric pump (Egnell breast-pump, Egnell Inc., Cary, Ill., USA) at 7 a.m. on days 2 and 4. Samples were stored at -70°C until analysis. Each milk sample was separated into three fractions: lipids, precipitable proteins and milk serum. To obtain the lipid fraction, samples were centrifuged at 2,300 g for 20 min at 4°C . The fat layer was removed and stored at -70°C until analysis. Although we did not test the milk for contamination, the literature reports that it contains cell membranes from fat globules, liposoluble vitamins and some sterols [14, 15]. The precipitable protein and the milk

Table 1. Composition and ^{13}C enrichment of experimental diets

	C4 diet based on corn (higher ^{13}C content)		C3 diet based on wheat (lower ^{13}C content)	
	day 1	day 2	day 3	day 4
Energy				
kcal/day	2,354±280	2,371±245	2,414±259	2,354±279
kcal/kg body weight	43.0±2.3	43.5±4.1	44.2±3.4	44.2±2.9
Carbohydrate, % of energy	70.9±1.4	72.8±1.2	71.2±0.8	70.0±0.3
Protein, % of energy	10.8±0.3	10.8±0.5	11.5±0.5	11.9±0.4
$\delta^{13}\text{C}$ vs. PDB in dietary proteins		-20.5±0.6		-23.2±0.3
Lipid, % of energy	19.4±0.7	18.0±1.0	17.9±0.6	18.3±0.4
$\delta^{13}\text{C}$ vs. PDB in dietary lipids		-18.1±1.4		-20.3±0.82
$\delta^{13}\text{C}$ vs. PDB in diet composites		-17.5±0.5		-25.2±0.3 ^a

Values are means ± SD.

^a Different from day 2 ($p < 0.01$).

serum fractions were obtained by adding 1 ml of a 10% solution of sodium tungstate and 1 ml of 0.6 N of H_2SO_4 per milliliter of defatted milk, heated at 80°C for 5 min and then centrifuged at 1,500 g for 5 min. The upper aqueous phase was considered as the serum milk fraction and the pellet as the precipitable protein fraction; both were stored at -70°C until analysis.

Breath Samples

Breath samples were collected in Douglas bags at hourly intervals from 7 a.m. on day 1 until midnight (12 p.m.) on day 4. Aliquots were transferred into evacuated glass tubes (Exetainer, Labco, High Wycombe, UK) and stored at 4°C until analysis.

Isotope-Ratio Mass Spectrometry of Breath Air, Diet and Breast Milk Fractions

The ^{13}C enrichment of CO_2 in breath air was determined by isotope-ratio mass spectrometry (Breath MAT, Finnigan MAT, Bremen, Germany), after chromatographic purification of the CO_2 in a continuous-flow inlet system, as previously described [16].

The samples of diet and milk fractions were combusted in sealed evacuated quartz capillary tubes at 900°C for 2 h and at 650°C for 1 h in the presence of cupric oxide and metallic silver and copper. The CO_2 produced by such combustion was purified cryogenically and the $^{13}\text{C}/^{12}\text{C}$ ratio was measured in a mass spectrometer model Finnigan Mat 250 (Finnigan MAT).

The $\delta^{13}\text{C}$ values were calculated by relating the $^{13}\text{C}/^{12}\text{C}$ ratio of the sample to the PDBV international standard ($\delta^{13}\text{C}_{\text{PDBV}}$) [17]. The $^{13}\text{C}/^{12}\text{C}$ values come from CO_2 generated by a phosphoric acid reaction with the VPDV international standard (PDBV is an acronym for Pee Dee belemnite, sold by IAEA in Vienna and VPDV is a carbonate of marine origin from a cretaceous formation) [17]. The $\delta^{13}\text{C}$ value was calculated by dividing the $^{13}\text{C}/^{12}\text{C}$ ratio value of each sample by the value provided by the PDBV international standard minus 1 and this was all multiplied by 1,000, using the following value for $^{13}\text{C}/^{12}\text{C}$:

$$^{13}\text{R}_{\text{PDBV}} = ^{13}\text{C}/^{12}\text{C} = 0.01117960.$$

Ignoring the uncertainty, the equation is defined by

$$\delta^{13}\text{C}_{\text{VPDB}} = \left[\frac{\left(^{13}\text{C} / ^{12}\text{C} \right)_{\text{Sample}}}{\left(^{13}\text{C} / ^{12}\text{C} \right)_{\text{VPDB}}} - 1 \right] \cdot 10^3.$$

This determination was made on the isotope-ratio mass spectrometer.

Statistical Analysis

Differences between means for descriptive variables were analyzed by paired t test and differences among mean $\delta^{13}\text{C}_{\text{PDBV}}$ values by t test for repeated measurements. Statistical significance was set at $p < 0.05$. All values are presented as mean ± SD. The SPSS Statistical Software program v. 8 (SPSS Inc., Chicago, Ill., USA) was used for statistical data analysis.

Results

There were no differences in the energy intake and macronutrient distribution between the two controlled diets or on the different days of the study (table 1).

^{13}C Enrichment in Breath Samples

The enrichment of ^{13}C in breath CO_2 on days 1 and 2 on the C4 diet is shown in figure 1. The $\delta^{13}\text{C}_{\text{PDBV}}$ in breath CO_2 increased progressively from the first hours of the morning (-15.4 $\delta^{13}\text{C}_{\text{PDBV}}$) until midnight (-13.2 $\delta^{13}\text{C}_{\text{PDBV}}$). There were important peaks and troughs in the $\delta^{13}\text{C}_{\text{PDBV}}$ of expired $^{13}\text{CO}_2$ across the day; the peaks were roughly associated with meals and the troughs were associated with fasting periods.

Fig. 1. Mean $\pm$ SD of the $\delta^{13}\text{C}_{\text{PDBV}}$ in breath CO_2 on days 1 and 2. Subjects ($n = 6$) were eating a C4-controlled diet, higher in ^{13}C , based on corn products and cane sugar. The mean $\delta^{13}\text{C}_{\text{PDBV}}$ increased progressively from the first hours of the morning ($-15.4 \delta^{13}\text{C}_{\text{PDBV}}$) until midnight ($-13.2 \delta^{13}\text{C}_{\text{PDBV}}$). There were peaks and troughs in $\delta^{13}\text{C}_{\text{PDBV}}$ of breath CO_2 during the day; peaks were roughly associated with meals and troughs were associated with fasting periods.

Fig. 2. Mean $\pm$ SD of the $\delta^{13}\text{C}_{\text{PDBV}}$ in breath CO_2 on days 3 and 4. There was a decline of 55.8% in the mean $\delta^{13}\text{C}_{\text{PDBV}}$ in breath CO_2 (from -13.9 to $-21.8 \delta^{13}\text{C}_{\text{PDBV}}$) on day 3 of the study, after switching to the C3 diet, which is lower in ^{13}C . After this, it remained stable at around $-20.0 \delta^{13}\text{C}_{\text{PDBV}}$ for the rest of the study. Peaks and troughs roughly associated with meals and fasting periods, respectively, were not noticeable.

Table 2. Mean $\delta^{13}\text{C}$ versus PDB in breast milk fractions

	Corn-based, higher ^{13}C diet	Wheat-based, lower ^{13}C diet
Breast milk		
Precipitable protein	-15.58 ± 0.43	-17.32 ± 1.52
Lipid	-19.28 ± 0.73	-23.93 ± 0.36^a
Milk serum	-14.14 ± 0.76	-20.11 ± 4.73^a

Values are means $\pm$ SD.

^a Different from the corn-based diet ($p < 0.01$).

On day 3 of the study, the first day of the C3 diet based on wheat and bee honey, there was a decline of 55.8% of the $\delta^{13}\text{C}_{\text{PDBV}}$ in breath CO_2 (from -13.9 to $-21.8 \delta^{13}\text{C}_{\text{PDBV}}$) in the first 16 h after changing to this diet. After this, it remained stable at around $-20.0 \delta^{13}\text{C}_{\text{PDBV}}$ for the rest of the study (fig. 2). Absorptive and postabsorptive peaks and troughs were not noticeable.

^{13}C Enrichment in Milk Fractions and Diet Composite Samples

The $\delta^{13}\text{C}_{\text{PDBV}}$ in two out of the three milk fractions (lipid and milk serum) obtained during the C4 diet was significantly higher than in the corresponding fractions of milk collected while subjects were on the C3 diet ($p < 0.01$; table 2).

The $\delta^{13}\text{C}_{\text{PDBV}}$ value was significantly higher in the composites of the C4 diet ($-17 \delta^{13}\text{C}_{\text{PDBV}}$) than in the C3 diet ($-25.2 \delta^{13}\text{C}_{\text{PDBV}}$; $p < 0.001$). The enrichment of ^{13}C in the dietary protein was similar in the two diets (table 2).

Discussion

We present evidence that individuals on diets based on C4 plant products (e.g. corn, sugar cane and sesame seed oil) have greater $\delta^{13}\text{C}_{\text{PDBV}}$ in their breath CO_2 than when on a diet based on C3 plant products (e.g. wheat, bee honey, citreous fruits and apples).

The high value of $\delta^{13}\text{C}_{\text{PDBV}}$ that prevailed, even during the fasting periods of days 1 and 2, must be attributed to the oxidation of nutrients not only after their immediate intestinal absorption, but also of nutrients mobilized from the fast-mobilizable pools. This suggests that it is plausible to measure flows of specific nutrients from body deposits, fat and glycogen. There are several studies that have successfully used nutrients that were naturally [9, 10, 18, 19] or artificially [20, 21] labeled for physiological examination.

It is clear that the ^{13}C enrichment of dietary fat and protein changed very little, thus most of the ^{13}C must have come from the dietary carbohydrates as shown by the $^{13}\text{CO}_2$ expired in the breath air and milk serum. It was not possible to measure the ^{13}C enrichment of dietary carbohydrates due to technical problems in separating the simple sugars and complex carbohydrates and fiber. The intake of fiber was approximately 20 g/day.

We are aware that the design of this study meant that the exhaled $^{13}\text{CO}_2$ represents the final oxidation of all macronutrients contained in the diet. However, the occurrence of such extreme changes in this experiment suggests that it could be used to study changes in the dietary habits of populations, particularly those in a transition from traditional diets to diets that include products with more C3 plant products, as in the case of industrialized food. This applies to several developing countries like Mexico in Central America and some of the countries in West Africa that are switching from corn-based traditional diets to more Westernized diets that are based on refined wheat-flour products as their main staple.

The changes presented here in the $\delta^{13}\text{C}_{\text{PDBV}}$ value of the two human milk fractions (lipids and milk serum)

demonstrate the rapid changes in the ^{13}C enrichment of several nutrients (contained in the fractions) that occur as a consequence of changes in the diet. The milk serum and lipid fractions had the greatest changes in magnitude whereas the precipitable protein remained almost unchanged. The most abundant molecule in milk serum, by far, is lactose [22, 23]. So most of the changes in the $\delta^{13}\text{C}_{\text{PDBV}}$ in this fraction should be attributed to the ^{13}C glucose from the diet, the main substrate for the synthesis of lactose. One example of studies in this field is the experiment reporting the incorporation of exogenous ^{13}C lactose, administered orally to lactating women, into milk oligosaccharides, and its excretion through the urine of their infants [24]. The lipid fraction of the milk contains large amounts of saturated fat that may come from the maternal liver or from mammary synthesis, with glucose as a substrate [22]. The changes in milk fat ^{13}C enrichment are more due to changes in the ^{13}C content of glucose.

In summary, we present evidence that subjects habitually consuming a diet based on C4 plants have a larger body content of ^{13}C -labeled metabolites. Changing the dietary content with respect to intake C3/C4 plants, it is feasible to assess metabolic pathways in physiological studies. Further studies focusing on individual nutrients are warranted.

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