Differential Biohydrogenation and Isomerization of [U-^{13}C]Oleic and [1-^{13}C]Oleic Acids by Mixed Ruminal Microbes

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ABSTRACT: The additional mass associated with ^{13}C in metabolic tracers may interfere with their metabolism. The comparative isomerization and biohydrogenation of oleic, [1-^{13}C]oleic, and [U-^{13}C]oleic acids by mixed ruminal microbes was used to evaluate this effect. The percent of stearic, *cis*-14 and -15, and *trans*-9 to -16 18:1 originating from oleic acid was decreased for [U-^{13}C]oleic acid compared with [1-^{13}C]oleic acid. Conversely, microbial utilization of [U-^{13}C]oleic acid resulted in more of the ^{13}C label in *cis*-9 18:1 compared with [1-^{13}C]oleic acid (53.7 vs. 40.1%). The isomerization and biohydrogenation of oleic acid by ruminal microbes is affected by the mass of the labeled tracer.

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Metabolic studies often use chemical tracers to evaluate *in vivo* events. A common method to create metabolic tracers is to replace an atom of the tracee molecule with a radioactive or stable isotope of that atom. These types of isotopes are less abundant in nature and are therefore easily distinguished from the isotope that is naturally more abundant (1). A summary of several studies has established that the use of tracer and higher doses of stable isotopes *in vivo* present no identifiable risk to human subjects (2). Therefore, *in vivo* measurements of FA metabolism may be obtained from the analysis of ^{13}C-labeled FA. Today, the use of stable isotopes to evaluate FA metabolism in humans is not uncommon (3,4). However, there is concern that not all isotopically labeled molecules are utilized equally. For example, different isotopically labeled glucose molecules are metabolized to varying extents (5,6). Furthermore, position and number of ^{14}C in labeled FA has been found to impact the extent of β-oxidation (7).

Previous research (8) has illustrated that [1-^{13}C]oleic acid is isomerized and biohydrogenated by ruminal microbes. Using these results as a model, it was hypothesized that there was no difference in the utilization of unlabeled oleic, [1-^{13}C]oleic, and [U-^{13}C]oleic acids by ruminal microbes despite the increased mass of the ^{13}C-labeled oleic acid molecules. Mixed ruminal microbes are necessary to carry out all possible isomerization and biohydrogenation processes (9). Additionally, ruminal microbes do not catabolize, elongate, or desaturate external FA to any great extent (10). Therefore, this model will only focus on differences in the isomerization and biohydrogenation of the treatment FA of differing masses without altering the isotope distribution in other metabolic pools.

EXPERIMENTAL PROCEDURES

Reagent grade oleic acid (97%) was purchased from Acros Organics (Geel, Belgium). [1-^{13}C]Oleic acid (99%) and [U-^{13}C]oleic acid (99%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All solvents were HPLC or GC grade. Dimethyl disulfide (DMDS) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Anhydrous ethyl ether, iodine, and sodium thiosulfate were purchased from Fisher Scientific (Pittsburgh, PA).

Microbial biohydrogenation of oleic acid was studied in cultures of microbes taken from the rumen of cattle as previously described (8). Cultures containing unlabeled, [1-^{13}C]-labeled, or [U-^{13}C]-labeled oleic acid were run in triplicate at 39°C under anaerobic conditions. Samples (5 mL) were taken from each culture at 0 and 48 h and immediately frozen. The samples were freeze-dried and then methylated using a two-step sodium methoxide and methanolic HCl procedure (11).

DMDS adducts of the FAME were prepared (8). The FAME and DMDS were analyzed by GC-MS (Agilent Technologies 6890N GC equipped with a 30 m × 0.25 mm with 0.2-μm film DB-225ms capillary column [Agilent J&W Scientific] and a 5973 inert series quadrupole mass-selective detector controlled by MSD ChemStation software [D.01.02.16] in the scan mode). For the FAME samples, column temperature was programmed from 50°C to 220°C at a rate of 6°C/min and held for 15 min. For the DMDS samples, column temperature was programmed from 195°C to 230°C at a rate of 0.5°C/min. Additionally, FAME were analyzed on a GC to determine the FA profile (12).

In order to measure the change in the ratio of ^{13}C to ^{12}C, the tracer (^{13}C) to tracee (^{12}C) ratio (TTR) for each FA was calculated from the mass abundance of the ^{13}C ($M + n$) and ^{12}C (M)

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Abbreviations: DMDS, dimethyl disulfide; E, enrichment; TTR, tracer to tracee ratio.

fragments using the equation TTR = $(M + n)/M$, where n is the number of ^{13}C atoms present in the fragment (1). In order to account for the natural levels of ^{13}C, the average TTR of samples taken from the unlabeled oleic acid cultures was subtracted from the TTR of samples from the ^{13}C-labeled oleic acid cultures, and the result was then adjusted for spectrum skew (1).

ANOVA using the GLM procedure of SAS (v. 9.1, SAS Institute, Inc., Cary, NC) was utilized to evaluate the ^{13}C enrichments at 0 and 48 h. Also, in an effort to evaluate the response to isotopic label, the degrees of freedom were partitioned into linear contrasts. All results are expressed as least square means. Additionally, ANOVA with the main effect of time on the percentage of each FA originating from oleic acid at 48 h and the distribution of the ^{13}C label at 48 h was evaluated for the [1-^{13}C]oleic and [U-^{13}C]oleic acid treatments. Least square means for each FA by treatment were analyzed for differences using Fisher's least significant difference with significance declared at $P \leq 0.05$.

RESULTS

Oleic acid was isomerized to a variety of *cis*- and *trans*-18:1 isomers and biohydrogenated to stearic acid (18:0) at the end of 48 h (Fig. 1). The other sources of oleic acid and its isomerization and biohydrogenation products in the microbial cultures (from the rumen inoculum and hay substrate) were minimal, and only linoleic (18:2 n-6) and linolenic (18:3 n-3) acids were considered in the data analysis. As expected, no enrichment was detected at either 0 or 48 h in the unlabeled oleic acid treatment. Excluding the minor contamination of [1-^{13}C]*trans*-9 18:1 (<1%) in the [1-^{13}C]oleic acid treatment and [U-^{13}C]*cis*-11 18:1 (5.5%) in the [U-^{13}C]oleic acid treatment, only oleic acid was enriched at 0 h in these two treatments (data not shown). Enrichment of *cis*-11 18:1 only occurred in the [U-^{13}C]oleic acid treatment. Therefore, only the change in weight percentage of *cis*-11 18:1 was evaluated.

The percentage of 18:0 and 18:1 isomers originating from oleic acid at 48 h was also evaluated. Excluding *cis*-9 and *cis*-

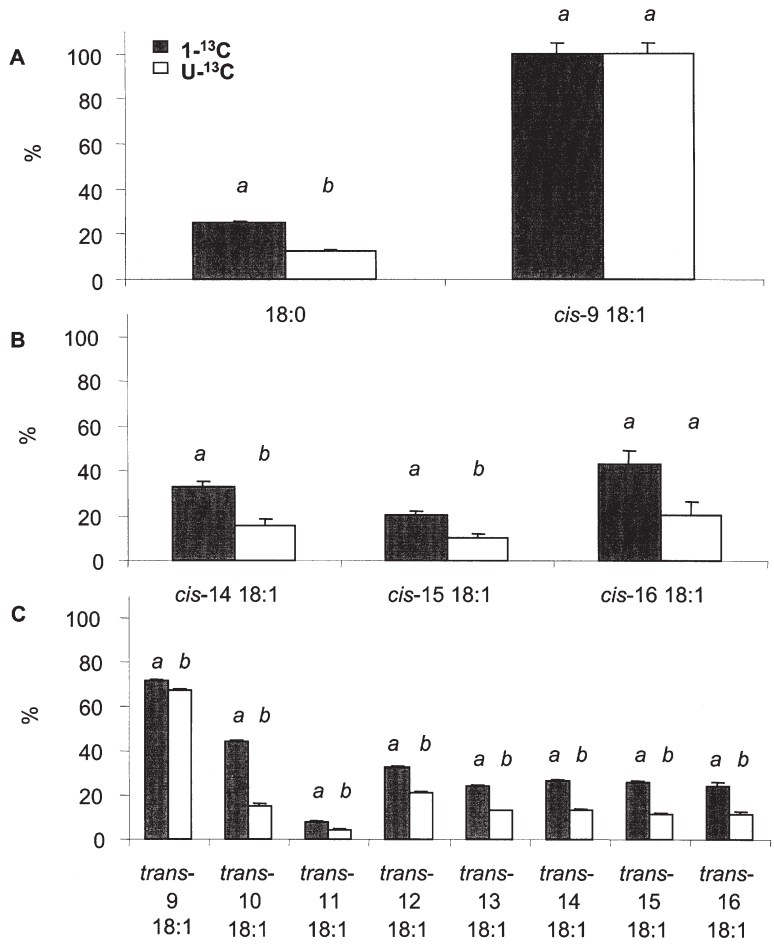

FIG. 1. Percent of (A) stearic and oleic, (B) *cis*-18:1, and (C) *trans*-18:1 FA originating from stably labeled oleic acid in microbial cultures. Determination occurred 48 h after [1-^{13}C]oleic (1-^{13}C) or [U-^{13}C]oleic (U-^{13}C) acid was added (25 mg per culture) to flasks. Values are mean ± SEM ($n = 3$). The SEM for 18:0 and *trans*-13 18:1 are too small to be viewed on figure. Columns with different letters differ ($P < 0.05$) within each FA variable.

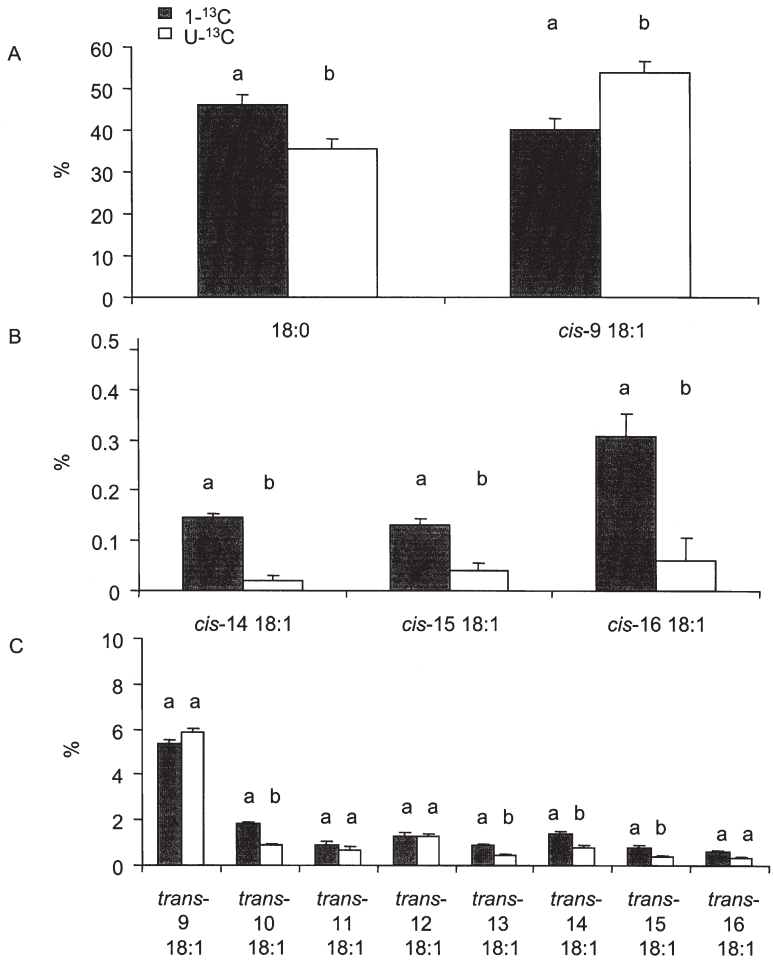

FIG. 2. Relative distribution of ^{13}C label from oleic acid in (A) stearic and oleic, (B) *cis*-18:1, and (C) *trans*-18:1 FA in microbial cultures 48 h after addition of [1-^{13}C]oleic (1-^{13}C) or [U-^{13}C]oleic (U-^{13}C) acid (25 mg per culture) to flasks. Values are mean ± SEM (n = 3). Columns with different letters differ ($P < 0.05$) within each FA variable.

16 18:1 isomers, there was a lower percentage of all other FA originating from oleic acid at 48 h for the [U-^{13}C]oleic acid treatment compared with the [1-^{13}C]oleic acid treatment (Fig. 1). Furthermore, when the overall distribution of the ^{13}C label was examined, treatment differences were observed (Fig. 2). A greater percentage of label remained in *cis*-9 18:1 in the [U-^{13}C]oleic acid treatment, whereas more of the label was detected in 18:0 and 18:1 isomers (*cis*-14, *cis*-15, *cis*-16, *trans*-10, *trans*-13, *trans*-14, and *trans*-15) in the [1-^{13}C]oleic acid treatment.

At 48 h, the weight percentage of 18:0 and most *cis*- and *trans*-18:1 FA increased (Table 1). The starting proportions of 18:0 in the cultures were variable among cultures; but no significant effect of isotopic label was detected. The *cis*-9 18:1 (oleic acid) weight percentage decreased over time; however, this was only a tendency when the singly and uniformly labeled oleic acid treatments were compared. The variation resulting from the contamination of the [U-^{13}C]oleic acid treatment with [U-^{13}C] *cis*-11 18:1 resulted in no differences observed when the unlabeled treatment was compared with the isotopically labeled treatments. However, when the isotopically labeled treatments were compared, differences in the concentration of [U-^{13}C]*cis*-11 18:1 were detected. Furthermore, differences due to addition of isotopic label and number of isotopes resulted in changes in the concentrations of several of the *cis*- and *trans*-18:1 FA (Table 1).

DISCUSSION

In our experiment, oleic acid was converted to stearic acid and a multitude of *cis*- and *trans*-18:1 isomers as previously described (8). Despite contamination of the ^{13}C FA sources, the formation of *trans*-18:1 monoenes from oleic acid is in agreement with previous research (8). It is unknown whether the *cis*- and *trans*-18:1 monoenes that are formed from oleic acid are intermediates or if they are end products of microbial manipulation of oleic acid. However, *trans*-9 18:1 was extensively intra-isomerized to other *cis*- and *trans*-18:1 and hydrogenated to stearic acid (13). Therefore, isomerization of oleic acid may be followed by further isomerization or hydrogenation to stearic acid.

TABLE 1
Proportions of Stearic Acid (18:0) and 18:1 FA Detected at 0 and 48 h when Oleic, [1-^{13}C]Oleic, or [U-^{13}C]Oleic Acids Were Added (25 mg) to Microbial Cultures[a]

	Treatment								
	Oleic		[1-^{13}C]Oleic		[U-^{13}C]Oleic			P^b	
Fatty acid	0 h	48 h	0 h	48 h	0 h	48 h	SE	1	2
18:0	6.57	65.23	10.39	63.19	11.95	66.23	1.39	NS	NS
18:1 Isomers									
cis-9	84.08	12.32	81.07	13.67	73.61	12.75	1.87	NS	0.07
cis-11	0	2.06	0	2.01	4.24	2.53	0.86	NS	0.03
cis-12	0	0.34	0.21	0.27	0	0.27	0.04	NS	0.02
cis-13	0.81	0.16	0.53	0.08	0.80	0.09	0.04	0.03	0.02
cis-14	0	0.02	0.02	0.15	0	0.03	0.01	<0.01	<0.01
cis-15	0	0.02	0.06	0.22	0.04	0.09	0.01	<0.01	<0.01
cis-16	0	0.07	0.04	0.25	0	0.67	0.02	0.06	<0.01
trans-5	0	1.17	0	0.92	0.07	0.84	0.03	<0.01	NS
trans-6	0	0.62	0	0.55	0	0.44	0.01	<0.01	<0.01
trans-7/8	0	3.63	0	3.09	0	2.66	0.11	<0.01	0.10
trans-9	0.34	2.32	0.47	2.56	0.52	2.05	0.06	NS	<0.01
trans-10	0	1.61	0	1.45	0	1.40	0.04	0.03	NS
trans-11	0.76	3.61	0.91	3.76	0.95	3.76	0.10	NS	NS
trans-12	0.06	1.62	0.17	1.36	0	1.39	0.10	NS	NS
trans-13	0.06	0.89	0.16	1.23	0.07	0.84	0.08	NS	0.02
trans-14	0.01	1.60	0.10	1.80	0	1.38	0.12	NS	0.07
trans-15	0.04	0.54	0.08	1.05	0	0.73	0.07	0.03	0.04
trans-16	0.51	0.38	0.08	0.86	0.20	0.70	0.21	NS	NS
18:2 n-6	2.89	0.92	2.63	0.76	3.58	0.87	0.06	NS	<0.01
18:3 n-3	3.88	0.87	3.11	0.76	3.97	0.90	0.10	0.09	<0.01

[a]The quantity of each FA is given as mg/100 mg of the FA presented. Values are the mean of three replicates at each time.
[b]1, oleic acid vs. the mean of [1-^{13}C]oleic acid and [U-^{13}C]oleic acid treatments; 2, [1-^{13}C]oleic acid vs. [U-^{13}C]oleic acid treatment; NS, nonsignificant.

Uniform, multiple, or single isotopically labeled FA are used to examine FA metabolism *in vivo* in humans and animals. Early milk fat researchers used isotopically labeled FA to examine the contribution of dietary FA to milk fat (14,15). Additionally, ^{2}H-labeled FA have been used to investigate the flow of FA through various plasma lipid classes in lactating women (16) and to determine the impact of FA position and structure of dietary triglycerides on absorption (17). However, there is limited research on the comparative utilization of stably labeled lipid and FA tracers of varying mass. When [23,24,25,26,27-^{13}C]cholesterol, [26,26,26,27,27,27-^{2}H]cholesterol, and [4-^{14}C]cholesterol were given intravenously and orally to healthy adult men and women, no differences in metabolism were observed (18). Similarly, when sample dilution correction factors were applied, no differences were detected in utilization of [17,17,18,18,18-^{2}H]linolenic, [U-^{13}C]linolenic, [17,17,18,18,18-^{2}H]linoleic, and [U-^{13}C]linoleic acids after simultaneous oral administration to rats (19). However, in isolated mitochondria, β-oxidation of [16-^{14}C]palmitic and [U-^{14}C]palmitic acids was reduced compared with [1-^{14}C]palmitic acid (7). Using mixed microbial cultures, slight differences were observed between the metabolism of unlabeled and isotopically labeled oleic acid. Furthermore, differences were also observed when singly or uniformly labeled oleic acid were compared (Table 1). These alterations in metabolism of isotopically labeled oleic acid by mixed ruminal microbes may be simply due to increases in the mass of the molecule or possibly due to some other isotope effect.

Despite differences in the extent of isomerization and biohydrogenation between the [1-^{13}C]oleic and [U-^{13}C]oleic acids, the same FA were enriched with ^{13}C. Additionally, the spectral shift associated with the use of [U-^{13}C]oleic acid is detected with less difficulty, as the mass fragments produced by MS analysis do not occur in the unlabeled oleic acid, but those produced by [1-^{13}C]oleic acid do occur. For example, the major spectral fragments 217 and 218 are used to identify FAME-DMDS derivatives of oleic and [1-^{13}C]oleic acids, respectively. Both of these fragments occur in the mass spectra of the FAME-DMDS derivative of unlabeled oleic acid. However, the major spectral fragment of [U-^{13}C]oleic acid is 226, which is a negligible fragment of oleic and [1-^{13}C]oleic acid. Furthermore, the detection of isotope discrimination may be trivial in mechanistic evaluations due to the relatively small differences observed. In the current study the entire FA pool is saturated with the labeled isotope, resulting in enrichment values of ^{13}C based on TTR calculations >100%. This large incorporation of labeled mass into the FA pool is not considered a tracer dose. For example, average *in vivo* measures of enrichment based on the TTR calculation in one study were <10% (12) when tracer doses of the labeled FA are used. Thus, there would be a considerable shift in the isotopic pool size being measured in the current study compared with other *in vivo* experiments.

The use of isotopically labeled FA has proven beneficial to research in lipid metabolism. The development of stable isotope tracers is necessary to safely study *in vivo* FA utilization in humans and other animals. However, researchers should consider the possible alterations in substrate utilization that result from some isotopically labeled compounds.

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