Biohydrogenation of Linolenic Acid to Stearic Acid by the Rumen Microbial Population Yields Multiple Intermediate Conjugated Diene Isomers

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Abstract

The current literature suggests that linolenic acid biohydrogenation converts to stearic acid without the formation of CLA. However, a multitude of CLA were identified in the rumen that are generally attributed to linoleic acid biohydrogenation. This study used a stable isotope tracer to investigate the biohydrogenation intermediates of \(^{13}\)C-linolenic acid, including CLA. A continuous culture fermenter was used to maintain a mixed microbial population obtained from the rumen of cattle at pH 6.5 for 6 d. The mixed fermenter contents were then transferred to batch cultures containing either \(^{13}\)C-labeled or unlabeled linolenic acid, which were run in triplicate for 0, 3, 24, and 48 h. The \(^{13}\)C enrichment was determined by GC-MS. After 48 h of incubation, 8 CLA isomers were significantly enriched, suggesting that these CLA isomers originated directly from linolenic acid. The cis-10, cis-12 CLA isomer exhibited the highest enrichment (21.7%), followed by cis-9, cis-11 and trans-8, trans-10 CLA. The enrichment of these 2 CLA isomers ranged from 20.1 to 21.1% and the remaining 5 CLA including cis-9, trans-11 CLA were <15.0%. A multitude of nonconjugated and partially conjugated 18:2 and 18:3 isomers was enriched during the 48 h of incubation. The results of this study confirm that mixed ruminal microbes are capable of the formation of several CLA and 18:3 isomers from linolenic acid, indicating that linolenic acid biohydrogenation pathways are more complex than previously reported. J. Nutr. 141: 1445–1450, 2011.

Introduction

Mammals are not capable of de novo synthesis of linolenic acid (cis-9, cis-12, cis-15 18:3), because they lack fatty acyl-CoA desaturase (12 and 15) activity. Linolenic acid is a precursor to EPA and DHA, which are involved in a multitude of biological functions, including inflammation, immunity, and anticonvulsant management (1,2). In ruminants, dietary linolenic acid rapidly disappears in the rumen stomach compartment through a process called biohydrogenation carried on by the anaerobic microbial population. Trienoic, dienoic, and monoenoic trans fatty acids are produced as intermediates during biohydrogenation of linolenic acid yielding stearic acid as the final product. Linolenic acid initially is isomerized to a partially conjugated trienoic fatty acid (cis-9, trans-11, cis-15 18:3) by a \(\Delta\)12-cis, \(\Delta\)11-trans-isomerase (3). Next, the cis-9 double bond is hydrogenated to produce a nonconjugated dienoic fatty acid (trans-11, cis-15 18:2), followed by hydrogenation of the trans-11/cis-15 double bond to produce a trans monoenoic fatty acid. These 18:1 isomers are further hydrogenated to produce stearic acid. Three additional nonconjugated 18:3 isomers (cis-9, trans-12, cis-15; cis-9, trans-12, trans-15; and trans-9, trans-12, trans-15 18:3) were reported in duodenal contents from cattle (4), and a partially conjugated 18:3 isomer (cis-9, trans-13, cis-15 18:3) was reported as the initial intermediate of the biohydrogenation of linolenic acid (5).

According to most published pathways of biohydrogenation, linolenic acid is usually hydrogenated without the formation of CLA isomers. A recent study (5), however, suggested that 2 CLA isomers (cis-9, trans-11 and trans-13, cis-15 CLA) are produced through linolenic acid biohydrogenation, but no supporting evidence was provided. Due to its many reported physiological functions, determining whether CLA is produced from linolenic acid is relevant for both human health and animal performance.

The objective of this study was to establish the number and identity of CLA intermediates that originate from linolenic acid biohydrogenation. To complete this objective, linolenic acid was labeled with \(^{13}\)C and incubated in cultures of mixed ruminal microorganisms to trace carbon transfer to CLA.

Materials and Methods

Reagents. U-\(^{13}\)C-linolenic acid (99% chemical purity; 96% \(^{13}\)C isotopic purity) was purchased from Larodan. 1-\(^{13}\)C-linolenic acid (>99% chemical purity; 99% \(^{13}\)C isotopic purity) was purchased from Medical
Isotopes. Unlabeled linolenic acid (cis-9, cis-12, cis-15 18:3; 99% chemical purity) was purchased from Sigma-Aldrich Chemical. Reagents for general fatty acid analysis, including sodium methoxide, methanolic HCl, and hexane, were also purchased from Sigma-Aldrich Chemical.

**Overall study design.** Two experiments were run to examine the transfer of $^{13}$C from linolenic acid to CLA. In both studies, mixed microorganisms were taken from the rumen of a fistulated dairy cow and incubated in a single continuous culture fermenter for 6 d to adapt the microbial population to the diet (Table 1). Contents from the continuous culture were then added to batch cultures containing the same diet (Table 1) plus an injection of linolenic acid in ethanol (10 g/L). Preadapting microorganisms to the diet in continuous culture was done to minimize lag times of fatty acid metabolism in batch cultures. Study 1 had 18 batch cultures, with one-half receiving 1.50 mg unlabeled linolenic acid in ethanol and the other one-half receiving 0.75 mg unlabeled linolenic acid + 0.75 mg U-$^{13}$C-linolenic acid in ethanol. Three batch cultures were removed from the labeled and unlabeled treatments at 0, 3, and 24 h after introduction of the isolate. Study 2 had 24 batch cultures, with one-half receiving 1.80 mg unlabeled linolenic acid in ethanol and the other one-half receiving 0.90 mg unlabeled linolenic acid + 0.90 mg U-$^{13}$C-linolenic acid in ethanol. Three cultures were removed from the labeled and unlabeled treatments at 0, 3, 24, and 48 h after introduction of the isolate.

**Continuous culture.** Whole ruminal contents were collected from a ruminally fistulated Holstein cow 2 h after being fed a 50% forage/50% concentrate diet. All surgical and animal care protocols were approved by the Clemson University Animal Care and Use Committee. Within 20 min of collection, large particles were removed from the whole ruminal contents by filtration through 2 layers of cheesecloth and the filtrate containing the microbial population was transferred immediately to the laboratory in a sealed container. With constant stirring, the filtered ruminal inoculum (~750 mL) was then added to a dual-flow continuous culture fermenter that was modified in construction and operation from the design described by Teather and Sauer (6). The main modifications were a reconfigured overflow sidearm that angled downward at ~45° to facilitate emptying, a faster stirring rate (45 rpm) that still allowed stratification of particles into an upper mat, a middle liquid layer of small feed particles, a lower layer of dense particles, and a higher feeding rate (60 g/d). The culture was maintained for 6 d (5 d for adaptation and the last day for sampling).

A total of 60 g of diet (Table 1) was inoculated into the fermenter daily in 2 equal portions at 0800 and 1630 h. A buffer solution (7) was delivered continuously using a peristaltic pump to achieve a 0.10/h fractional dilution rate. Buffer pH was titrated daily with sufficient 6 mol/L NaOH or 3 mol/L HCl to maintain a pH of 6.5. The fermenter was continuously infused with CO$_2$ at a rate of 0.020 L/min to maintain anaerobic conditions. The temperature of the fermenter was held at 39°C by a circulating water bath. Culture samples were taken for volatile fatty acid (VFA) analysis on the last day at 0 (before the 0800 feeding) and at 2 and 4 h after feeding.

**Batch culture.** Contents in the continuous fermenter were completely stirred (100 rpm) on d 6 beginning at 1200 h and 2-mL samples were transferred to screw-capped culture tubes containing 100 mg of diet (Table 1), 8.0 mL of buffered medium, and 0.4 mL of sodium sulfite reducing solution (8). Linolenic acid treatments were injected in ethanol. The tubes were flushed with carbon dioxide and then tightly capped. Tubes were incubated in a water bath at 39°C until removed at pre-determined incubation times. To immediately kill the ruminal microorganisms and stop enzymatic activity at each sampling time, 0.4 mL 6 mol/L HCl was injected into the culture tubes. For the 0-h samples, the HCl was injected prior to addition of the microbial inoculant. The samples were immediately placed in an ice bath and later stored at −5°C.

**Fatty acid analysis.** After freeze-drying, sodium methoxide and methanolic HCl were added to the batch culture tubes for direct trans-esterification of fatty acids to methyl esters (9). An internal standard (2 mg heptadecanoic acid) also was added at the start of methylation to quantify fatty acid masses. Quantities of individual fatty acids present in the cultures were determined on a Hewlett-Packard 5890A gas chromatograph equipped with P-2380 fused silica capillary column (100 m x 0.25 mm) with 0.2-μm film thickness (Supelco). The conditions used were initially 140°C for 3 min with a ramp of 3.7°C/min up to 220°C holding for 20 min. Helium was used as the carrier gas at 20 cm/s and peak areas were determined by a flame-ionization detector.

The methyl esters also were analyzed for $^{13}$C enrichment by GC-MS using an Agilent 6890N gas chromatograph equipped with a 5973 quadrupole mass selective detector. The GC-MS was equipped with a 100-μm x 0.25-mm chrompack CP-Sil 88 column with a 0.20-μm film thickness. The carrier gas was helium at 20 cm/s with splitless injection. Column temperature was programmed initially at 140°C for 5 min with a ramp of 4°C/min up to 220°C for 20 min. For the T-13C-linoleic acid cultures in Expt. 1, the GC-MS used electron impact ionization in select ion mode. The ion chosen for analysis had a m/z of 87 in unlabeled cultures. The tracer-trace ratio (TTR) was calculated as the m/z of 88 to 87 in the labeled cultures minus the m/z of 88 to 87 in the unlabeled cultures to adjust for the natural occurrence of the $^{13}$C isotope. For the U-$^{13}$C-linoleic acid cultures in Expt. 2, the GC-MS was run in chemical ionization mode using methane as the reagent gas. The protonated quasi-molecular ion (M) was selected for analysis in select ion mode. The TTR was calculated as $|[(M + 18)/M]|$ in the labeled cultures − $|[(M + 18)/M]|$ in the unlabeled cultures. Results are presented as percent enrichment defined as TTR × 100.

The identity of double bond position and geometry were verified by comparison of peaks to purchased reference standards, combined with structural analysis by covalent adduct chemical ionization tandem MS done by J. T. Brenna at Cornell University according to methods previously outlined (10,11). Briefly, fatty acids were separated with a CP-Sil 88 capillary column (100 m x 0.32 mm x 0.25 μm) and temperature programmed from 80 to 120°C at a rate of 10°C/min and then increased to 220°C at a rate 2.5°C/min. Total run time was 60 min. Fatty acids eluting into the MS undergo an ion-molecule reaction to form an adduct with a molecular weight 54 mass units above that of the parent compound. Collisional dissociation of this adduct yields diagnostic ions that are characteristic of the double bond position. In the case of CLA, relative diagnostic ion intensity is indicative of double bond geometry (10) and can otherwise be discerned by GC relative retention time.

**Statistical analysis.** An initial step in the analysis was to determine whether the 2 experiments should be reported separately or combined into 1 report. To accomplish this step, a series of 2-sample t tests were performed. For instance, the percent enrichments of linoleic acid in the first experiment were 32.0, 31.6, and 30.7% (for 0, 3, and 24 h incubation) and in the second experiment the percent enrichments were 35.9, 36.1, and 36.6% (for 0, 3, and 24 h incubation). The absolute differences in percent enrichments between the 2 experiments were ≤6% at each hour of incubation and found to be not significantly different from zero. These series of tests revealed that there was no indication of an incubation time × experiment interaction. Given that there was no evidence of interaction, the results of the 2 separate experiments were combined giving n = 6 for all incubation hour percent enrichment means.

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**TABLE 1** Composition of the continuous and batch culture diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content (g/kg dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa pellet</td>
<td>500</td>
</tr>
<tr>
<td>Ground corn</td>
<td>242</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>109</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>121</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>13.5</td>
</tr>
<tr>
<td>Trace mineral salt</td>
<td>6.2</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>8.2</td>
</tr>
</tbody>
</table>

1 Contained (g/kg): NaCl, 955–9.8; Na, 10.0; Mn, 7.5; Fe, 6.0; Mg, 0.5; Cu, 0.32; I, 0.28, and Co, 0.11.
except for 3 h in Expt. 2 where n = 3. Note that combining experimental data when there is no evidence of interaction can occur when all incubation hours do not appear in both experiments.

The percent enrichment least squares mean ± SEM was calculated for each fatty acid. Intermediates were judged to arise from linolenic acid carbons if the average of enrichment means differed significantly from zero based on Student’s t test.

All calculations were performed using SAS (version 9.2). All significance tests were performed with α = 0.05.

**Results**

From 0 to 48 h of incubation in batch cultures, the linolenic acid concentration declined (P ≤ 0.05) and was accompanied by increases (P ≤ 0.05) in concentrations of trans-11 18:1 and stearic acid, indicating active biohydrogenation (Fig. 1). On a total culture pool basis, linolenic acid declined from 7.9 to 0.02 μmol/culture, trans-11 18:1 increased from 0.21 to 2.9 μmol/culture, and stearic acid increased from 1.5 to 6.7 μmol/culture. Losses over 48 h as a percentage of the original amounts were 97.3, 87.9, and 64.3% for linolenic, linoleic, and oleic acids, respectively. Reported in vivo ruminal losses for the same fatty acids were 96.3, 87.9, and 76.6%, respectively. Linolenic acid biohydrogenation, as depicted in most pathways.

Enrichment of all unidentified 18:2 isomers increased (P ≤ 0.05) over time in several 18:3 isomers, including the 2 that were identified as partially conjugated.

Eight CLA isomers could be positively identified in the culture contents, including details on double bond location and geometry. Three identified CLA isomers had mixed geometry, including cis-9, trans-11; trans-10, cis-12; and trans-9, cis-11 CLA (Fig. 2). Of the remaining 5 identified CLA isomers, 3 were all trans (trans-9, trans-11; trans-13, cis-10; and trans-9, cis-11) and 2 were all cis (cis-9, cis-11 and cis-10, cis-12). None of the CA isomers were enriched at 0 h, but enrichment was detected from 3 to 48 h of incubation. Enrichment declined (P ≤ 0.05) over time in several 18:3 isomers, including the 2 that were identified as partially conjugated.

| TABLE 3 | Enrichments of 18:2 and 18:3 isomers having limited or no structural information relative to double bond location or cis-trans geometry in batch cultures of ruminal contents transferred from a Holstein cow to a continuous culture and dosed with 13C-linoleic acid

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>0 h</th>
<th>3 h</th>
<th>24 h</th>
<th>48 h</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.12</td>
</tr>
<tr>
<td>Linoleic</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.32</td>
</tr>
<tr>
<td>Linolenic</td>
<td>33.5</td>
<td>33.3</td>
<td>33.1</td>
<td>35.5</td>
<td>0.34</td>
</tr>
<tr>
<td>Stearic</td>
<td>NS</td>
<td>NS</td>
<td>7.2</td>
<td>16.3</td>
<td>0.10</td>
</tr>
<tr>
<td>trans-11 18:1*</td>
<td>NS</td>
<td>2.0</td>
<td>9.1</td>
<td>21.1</td>
<td>0.15</td>
</tr>
</tbody>
</table>

1 Values are least square means and the pooled SEM, n = 6. 2 *Time effect, P ≤ 0.05.

3 ND, not fatty acid detected.

4 NS, not significantly different from zero, P > 0.05.

**FIGURE 1** Total fatty acid concentrations in batch cultures of ruminal contents transferred from a Holstein cow to a continuous culture during 48-h incubations. Values are least square means, n = 6. Pooled SEM was 0.007 for trans-11 18:1 and 0.004 for all other fatty acids.
This study was designed to identify intermediates of linolenic acid biohydrogenation that eventually could be used to build a detailed pathway for its biohydrogenation. Very few 18:3 intermediates have been recognized. Many descriptions of linolenic acid biohydrogenation include only a single 18:3 intermediate, usually cis-9, trans-11, cis-15 18:3 (3,16). The cis-9, trans-11, cis-15 18:3 and its trans-11, cis-15 18:2 conversion product are presumed to be produced by a 12-cis, 11-trans-isomerase similar to the one isolated from Butyrivibrio fibrisolvens (17).

Alternative pathways of 18:3(n-3) biohydrogenation have been proposed that include, in addition to cis-9, trans-11, cis-15 18:3, the formation of trans-10, cis-12, cis-15 (18) and cis-9, trans-13, cis-15 18:3 isomers (5). Although their formation from 18:3(n-3) biohydrogenation was not proven, these additional 18:3 isomers are commonly detected in meat and milk fat from ruminant species. For instance, the cis-9, trans-11, cis-15 18:3 was the major 18:3 isomer found in both muscle and milk lipid extracts from cattle tissue, whereas the cis-9, trans-13, cis-15 18:3 isomer was detected only in muscle lipid extract (19). More recently, cis-9, trans-11, trans-15 18:3 was characterized in milk fat from sheep (20), which the authors proposed could originate from the isomerization of the cis-15 bond in cis-9, trans-11, cis-15 18:3 arising from biohydrogenation.

In this study, enrichment was seen in a multitude of 18:3 isomers as early as 3 h of incubation. Because the primary objective was to identify the nature of CLA isomers formed from 18:3(n-3) biohydrogenation, the exact structural identity of the enriched 18:3 isomer was not determined. It was confirmed that 2 of the 14 18:3 enriched isomers were partially conjugated. Fully conjugated 18:3 has been of interest to researchers for over a decade as the precursor of CLA or for possible biological effects. The most frequently encountered fully conjugated 18:3 is the cis-9, trans-11, trans-13 18:3, followed by cis-8, trans-10, cis-12, trans-8, trans-10, cis-12, cis-9, trans-11, cis-13; and trans-9, trans-11, cis-13 18:3 (21). More recently, Shingfield et al. (13) summarized findings from a multitude of studies on biohydrogenation and proposed the formation of 3 18:3 isomers (cis-9, trans-11, trans-13; trans-9, trans-11, cis-15; cis-9, trans-13, cis-15) in the initial step of linoleic acid biohydrogenation. Two of the 3 isomers were partially conjugated. These reports support an initial isomerization step for linoleic acid biohydrogenation that yields a multitude of 18:3 isomers varying in double bond location and geometry, including some double bonds in conjugated positions.

Several nonconjugated 18:2 isomers originated from linolenic acid biohydrogenation, but identification of double bond position and geometry was not attempted. The trans-11, cis-15 18:2 is generally the major nonconjugated 18:2 isomer originating from linolenic acid in most depictions of biohydrogenation (22). This isomer is thought to arise from reduction of the cis-9 double bond in cis-9, trans-11, cis-15 18:3 by some microbial species such as B. fibrisolvens (23). Similarly, cis-9, trans-13 18:2 would be expected to arise from reduction of the cis-15 double bond in cis-9, trans-13, cis-15 18:3 (5). Moreover, cis-9, trans-13 18:2 may be endogenously produced in tissues by the 9-desaturation of trans-13 18:1, supporting the possibility that cis-9 trans-13 18:2 could be formed by both endogenous and ruminal biohydrogenation sources (24). The cis-9, trans-12 18:2; trans-9, trans-12 18:2, trans-9, cis-12 18:2; and cis-9, cis-15 18:2 isomers were also found in ruminal contents (22).

The major focus of this study was to determine whether linolenic acid biohydrogenation could lead to the formation of 1 or more CLA isomers. Many biohydrogenation pathways show linolenic acid being transformed to cis-9, trans-11, cis-15 18:3 by a microbial isomerase, which is then converted to trans-11, cis-15,
18:2 followed by hydrogenation of the trans-11/cis-15 double bond to form stearic acid. This pathway led many researchers to think that CLA are not formed through linolenic acid biohydrogenation. Recent results refute this claim and showed the synthesis of 2 CLA isomers, cis-9, trans-11 CLA and trans-13, cis-15 CLA, from linolenic acid biohydrogenation (5). In the present investigation, CLA synthesis from linolenic acid was confirmed with carbons from linolenic acid being traced to 8 CLA isomers. This does not preclude that additional CLA isomers might originate from linolenic acid, but additional isomers could not be separated and positively identified in this study.

In most situations, cis-9, trans-11 CLA is the predominant CLA isomer in ruminal contents, accounting for ~88% of the total CLA (22). In this study, the enrichment of cis-9, trans-11 CLA was ~14% at 48 h, suggesting that ~40% of the cis-9, trans-11 originated from linolenic acid biohydrogenation. Similar enrichments were seen for trans-10, cis-12 CLA, which is considered the second major CLA, and cis-9, trans-11 CLA, showing that the majority of these isomers originated from fatty acid sources other than linolenic acid. The cis-9, trans-11 CLA is produced from linoleic acid by B. fibrisolvens (25), and trans-10, cis-12 CLA by Propionibacterium acnes and Propionibacterium freudenreichii (26). These 3 CLA isomers can then be converted to trans-9, trans-11 CLA by isomerases identified in Bifidobacterium species (27).

Enrichments were detected in several other CLA isomers (trans-9, cis-11; cis-9, cis-11; trans-11, trans-13; trans-8, trans-10; and cis-10, cis-12 CLA) at 3–48 h. These are generally regarded as minor CLA isomers in ruminal contents. For instance, the cis-9, cis-11 CLA is typically present at ≤2% of total CLA isomers (24). Although they might comprise a smaller proportion of total CLA produced in the rumen, several (cis-9, cis-11; trans-8, trans-10; cis-10, cis-12) had the highest isotopic enrichments (>22%) among all CLA isomers, indicating that linolenic acid provided more than one-half the carbons for their synthesis.

The current study verified the formation of cis-9, trans-11 and trans-9, cis-12 CLA, plus 6 additional CLA isomers that contained 13C from the labeled linolenic acid added to the microbial cultures. These results indicate that CLA that accumulates in ruminal contents of cattle can at least partially originate from biohydrogenation of linolenic acid, despite the depiction in many published pathways that linolenic acid does not lead to CLA formation. The results of this investigation indicate that further revision is needed for the putative pathways of biohydrogenation proposed by Shingfield et al. (13) that presently include only 3 CLA as intermediates of linolenic acid biohydrogenation. The major CLA isomer in dairy products and ruminant fat is cis-9, trans-11 CLA, followed by trans-7, cis-9 CLA, which arise predominantly from endogenous synthesis involving Δ9-desaturase. Because linolenic acid is a common constituent of lipids in many animal feed ingredients and is the major unsaturated fatty acid in pasture, the contribution to CLA production should not be ignored, especially for less prominent CLA isomers having unknown physiological consequences.

Several points should be considered when attempting to extrapolate the results of the present in vitro study to the live animal. First, a total of 8 CLA isomers are reported in this study to arise from the biohydrogenation of linolenic acid. Possibly there were more, but the separation and structural identification of all peaks in the CLA range of the GC chromatogram, including many that were extremely small, was not attempted or even possible. The results, therefore, show CLA isomers that are most likely seen in ruminal contents when high-linolenic acid diets are consumed by ruminant species. A second point of interpretation is that this study does not prove that linolenic acid will yield all these CLA isomers in the live animal under all circumstances, but only proves the capability of the anaerobic population to convert linolenic acid to CLA. The production of CLA isomers in ruminal contents varies with the predominating microbial species such that changes in diet offered the animal could dictate the presence or absence of a particular CLA isomer.

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Literature Cited


