Linoleic Acid Conjugation by Human Intestinal Microorganisms Is Inhibited by Glucose and Other Substrates In Vitro and in Gnotobiotic Rats

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Beate Kamlage, Ludger Hartmann,1 Bärbel Gruhl and Michael Blaut

German Institute of Human Nutrition, Department of Gastrointestinal Microbiology, 14558 Potsdam-Rehbrücke, Germany

ABSTRACT The anticarcinogen conjugated linoleic acid (CLA) is a product of bacterial activity that isomerizes linoleic acid (LA) in the rumen of herbivores. Therefore, fatty dairy products in the human diet are enriched with CLA. Although bacteria capable of in vitro LA conjugation were detected in the human intestinal tract, LA synthesis from dietary sunflower seed oil was not observed in gnotobiotic rats associated with these intestinal bacteria. The objective of the study was to investigate variables that affect LA conjugation. In vitro, LA conjugation was strongly inhibited by glucose and other substrates. Concentrations of 1.5 mmol glucose/L inhibited LA conjugation by 50%. Methyl-alpha-D-glucoside was a less effective inhibitor than glucose, and 2-deoxy-D-glucose did not inhibit LA conjugation at all. To analyze the concentration of carbohydrates in intestinal contents, the LA-conjugating bacterial mixed culture and human fecal microorganisms were introduced into germ-free rats. Samples of feces and cecum and colon contents of both groups exhibited in vitro LA-conjugating activity. Rats associated with human intestinal microorganisms contained 5.7 ± 1.3 mmol glucose/L in the cecal contents and 6.6 ± 1.0 mmol glucose/L in the colonic contents. Rats associated with CLA-producing bacterial culture contained 3.4 ± 1.3 mmol glucose/L in the cecal contents and 4.2 ± 1.0 mmol glucose/L in the colonic contents. These values are within a range that may explain the observed inhibition of LA conjugation in vivo. J. Nutr. 130: 2036–2039, 2000.

KEY WORDS: • conjugated linoleic acid • gnotobiotic rats • glucose • inhibition • intestinal microorganisms

The isolated double bonds of linoleic acid (LA; 9c,12c-octadecadienoic acid) are isomerized to a conjugated system by bacteria in the rumen of herbivores (Kepler et al. 1966). A mixture of isomers of conjugated linoleic acid (CLA) is produced that differ with respect to the position of the double bonds (Δ9, Δ11, or Δ12) and the cis-trans stereochemistry (Ha et al. 1987, Yurawecz et al. 1998). Conjugated linoleic acid is absorbed by ruminants and enriched in milk fat and body fat up to 87 μmol CLA/g fat, depending on the feeding regimen (Kelly et al. 1998).

After anticarcinogenic activity of CLA was reported by Ha et al. (1987), several investigations established that CLA is an effective anticarcinogen, inhibiting skin, mammary and forestomach neoplasia in humans and rodents (Ha et al. 1990, Ip et al. 1997, Ip et al. 1999, Liu and Belury 1997). An antiatherogenic effect of CLA was postulated (Nicolosi et al. 1997), but questioned recently (Munday et al. 1999). Furthermore, reports of a decrease of body fat in mice has been published (Park et al. 1999).

Although most CLA in the human diet seems to be of bacterial origin, very little is known about the production of CLA by bacteria. In the rumen, Butyri vibrio fibrisolvens is the only known organism capable of CLA production (Kepler et al. 1966). Moreover, bacteria in the intestine of monogastric animals and humans are capable of CLA production. The increase in CLA concentration in various tissues of rats in response to feeding free LA was explained by the activity of intestinal bacteria (Chin et al. 1994). However, the underlying formation of CLA seems to be restricted to the feeding of free LA. In contrast to the situation in ruminants, the consumption of LA esterified to glycerol by humans and rats did not increase the amount of CLA in serum and various body tissues (Chin et al. 1994, Herbel et al. 1998). Recently, we demonstrated that gnotobiotic rats associated with a mixed bacterial culture capable of in vitro CLA formation did not accumulate CLA in various body tissues when fed a sunflower seed oil–fortified diet (Kamlage et al. 1999). This bacterial culture had been enriched from a fecal sample of a human volunteer as described recently (Kamlage et al. 1999). CLA production of the culture was highly oxygen sensitive and occurred only in the late stationary growth phase (after 90 h). Only free, nonesterified LA was a substrate of conjugation, and CLA was released into the medium and not incorporated into cell lipids.

Fecal samples from these rats exhibited in vitro LA-conjugating activity in contrast to fecal samples from the germ-free control group. The aim of this study was to identify conditions that inhibit the LA-conjugation activity of the bacterial mixed culture in vitro. The results of our experiments led to a new hypothesis that may explain the observed absence of in vivo LA-conjugation activity.

MATERIALS AND METHODS

CLA-producing mixed bacterial culture. Enrichment of the CLA-producing bacteria from a human fecal sample was described recently (Kamlage et al. 1999). The culture was maintained in rubber-stopped anaerobic cultivation tubes containing the following compounds (per L): 9 g tryptic peptone from meat, 1 g proteose peptone, 3 g meat extract, 4 g yeast extract, 3 g NaCl, 2 g NaHPO4, 0.5 mL Tween 80, 0.1 g MgSO4 · 7 H2O, 5 mg FeSO4 · 7 H2O, 3.4

1 To whom correspondence should be addressed.
mg MnSO₄ · 2 H₂O, 0.25 g L-cysteine · HCl, 0.25 g L-cystine, 10 mg hemin and 1 mg resazurin. The gas phase consisted of 80 vol% N₂ and 20 vol% CO₂, and the pH was adjusted to 6.8–7.2. After autoclaving, 4 mmol/L of filter-sterilized (0.2-μm sterile filter, Sartorius, Göttingen, Germany) LA was added (Roth, Karlsruhe, Germany). Cultures were shaken at 37°C on a rotary shaker (140 revolutions/min). After incubation for at least 90 h, samples were subjected to CLA analysis (see below).

**Experiments with growing cells.** To investigate the substrate inhibition of LA conjugation, concentrated anaerobic stock solutions of various mono-, di-, tri- and polysaccharides, aminosugars, sugar alcohols and pyruvate were sterilized by autoclaving (meso-erythritol, glycerol, meso-inositol, D-sorbitol, inulin, starch from potatoes) or filter-sterilized (N-acetyl-D-glucosamine, D-arabinose, D-fructose, D-galactose, D-glucose, D-glucomannose, D-mannose, D-ribose, D-xyllose, D-cellobiose, D-lactose, D-lactulose, D-maltose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, sodium fumarate, DL-sodium lactate, sodium pyruvate). Substrates were added to the medium described above to a final concentration of 33 mmol/L (monosaccharides, aminosugars, sugar alcohols and pyruvate), 16.5 mmol/L (disaccharides, fumarate and DL-lactate), 11 mmol/L (trisaccharides) and 1 g/L (polysaccharides), respectively. For each substrate, four parallel samples were inoculated with the CLA-producing mixed culture and shaken at 37°C on a rotary shaker. After incubation for 90 h, samples were subjected to CLA analysis (see below). Cultures without substrate served as controls.

The concentration-dependent inhibition of LA conjugation by glucose and the effects of the glucose analogs methyl-α-D-glucoside and 2-deoxy-D-glucose (Sigma, Deisenhofen, Germany) were investigated by inoculation of media containing increasing concentrations of filter-sterilized substrates from 1 to 20 mmol/L with the mixed bacterial culture.

**Animal experiment.** Specifications of the germ-free rat strain AVN-Ipcv-Wistar-Rehbrücke were given recently (Kamlage et al. 1999). The protocol for the animal experiment was approved by the Animal experiment. Specifications of the germ-free rat strain AVN-Ipcv-Wistar-Rehbrücke were given recently (Kamlage et al. 1999). The protocol for the animal experiment was approved by the Ministry of Nutrition, Agriculture and Forestry, Brandenburg, Germany.

Germ-free male rats (n = 12; 5 wk old), weighing 117 ± 7.5 g, were divided randomly into two groups. They were fed an irradiated pelleted diet (25 kGy) consisting of a commercial rat breeding diet (Bro®lio, Hamm, Germany) with the following composition (per kg): crude protein (225 g), crude fat (50 g), crude fiber (45 g), ash (65 g), moisture (135 g) and nitrogen free extract (480 g). The diet was supplemented with 60 g of sunflower seed oil (Bro®lio, Hamm, Germany) LA was added (Roth, Karlsruhe, Germany). Cultures were shaken at 37°C on a rotary shaker (140 revolutions/min). After incubation for at least 90 h, samples were subjected to CLA analysis (see below). Cultures without substrate served as controls.

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The lipids were saponified in duplicate and analyzed as described (Kunst et al. 1984). Controls received water instead of a solution containing 1.5 g Fe(NH₄)(SO₄)₂·6H₂O and 0.025 mol Na₂CO₃/L. Chlorophyll was determined enzymatically as described (Kunst et al. 1984).

**RESULTS**

**Inhibition of LA-conjugating activity.** Without any substrate added, concentrations of 0.2–1.4 mmol CLA/L were produced from 4 mmol LA/L by the bacterial mixed culture. In the presence of N-acetyl-D-glucosamine, D-arabinose, D-cellobiose, D-fructose, fumarate, D-galactose, D-glucosamine, D-glucose, glycerol, inulin, D-lactate, D-lactulose, D-maltose, D-mannose, D-melezitose, sodium pyruvate, D-raffinose, D-ribose, D-saccharose, D-sorbitol, starch, D-trehalose and D-xyllose, the CLA concentrations in the cultures did not exceed the CLA concentrations in the uninoculated controls. The growth of the bacteria was not inhibited by the substrates as evident from microscopic examinations. In contrast, meso-inositol, meso-erythritol and D/l-lactate did not inhibit CLA production of the culture. This indicated that a simple osmotic effect cannot explain the inhibition of LA conjugation by the various carbohydrates. It is not known whether the growth of the LA-conjugating species in the mixed bacterial culture was specifically repressed or whether the enzymatic CLA-producing activity was inhibited.

To investigate the inhibitory effect of glucose in more detail, media with increasing concentrations of glucose were inoculated with the CLA-forming culture and after incubation for 90 h, analyzed for CLA (Fig. 1A). In the presence of >2 mmol glucose/L, a significant (P < 0.01) inhibition of CLA production became evident. The CLA concentrations found in cultures with >5 mmol glucose/L were in the range of those measured in uninoculated controls, indicating that essentially no LA conjugation had occurred in these cultures. The glucose concentration that inhibited LA conjugation by 50% was calculated to be 1.5 mmol glucose/L.

Methyl-α-D-glucoside was a weaker inhibitor of LA conju-
CONCENTRATIONS OF GLUCOSE AND TOTAL REDUCING SUGARS AND ACTIVITIES OF LINOLEIC ACID CONJUGATION IN CEcum AND COLON CONTENTS AND FECES OF ASSOCIATED GNTOBIOTIC RATS

**TABLE 1**

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Glucose (mmol/L)</th>
<th>Total reducing sugars (μmol CLA/L)</th>
<th>Activity of LA conjugation (μmol CLA/L·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats associated with human fecal microorganisms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecum contents</td>
<td>5.7 ± 1.3</td>
<td>23.7 ± 2.8</td>
<td>1.15 ± 0.52</td>
</tr>
<tr>
<td>Colon contents</td>
<td>6.6 ± 1.0</td>
<td>27.4 ± 2.6</td>
<td>0.44 ± 0.28</td>
</tr>
<tr>
<td>Feces</td>
<td>ND</td>
<td>ND</td>
<td>1.01 ± 0.74</td>
</tr>
<tr>
<td>Rats associated with LA conjugating bacterial culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecum contents</td>
<td>3.4 ± 1.3</td>
<td>27.9 ± 4.8</td>
<td>3.69 ± 0.70</td>
</tr>
<tr>
<td>Colon contents</td>
<td>4.2 ± 1.0</td>
<td>29.5 ± 1.2</td>
<td>3.05 ± 1.13</td>
</tr>
<tr>
<td>Feces</td>
<td>ND</td>
<td>ND</td>
<td>2.15 ± 1.01</td>
</tr>
</tbody>
</table>

1 Germ-free rats were associated with human fecal microorganisms (n = 6) or with a LA-conjugating mixed bacterial culture enriched from human feces (n = 6). They were fed a diet enriched with sunflower seed oil for 4 wk. Cecum and colon contents of the rats were analyzed for glucose and total reducing sugars. Values are means ± SD.

2 LA, linoleic acid; CLA, conjugated linoleic acid; ND, not determined.
position of the microbial communities of the rumen on the one hand and the cecum and colon on the other hand. For instance, B. fibrisolvens, a ruminal bacterium capable of CLA synthesis (Kepler et al. 1966), is not a member of the bacterial culture studied in this investigation (Kamlage et al. 1999).

The exact mechanism of the glucose-mediated inhibition of LA conjugation is still unknown. Theoretically, glucose may have either inhibited the growth of the LA-conjugating bacteria in the mixed culture or the expression or activity of the LA-conjugating enzyme system. LA-conjugation activity of the bacterial mixed culture is dependent on the growth phase and occurs only in the late stationary phase (Kamlage et al. 1999). It is not yet known whether the metabolic conditions of the stationary growth phase in vitro are similar to the conditions in vivo when the bacteria grow inside the cecum or colon. We speculate that the restriction of LA-conjugating activity to the late stationary growth phase and the observed inhibition by glucose and other substrates were caused by the same mechanism. It is conceivable that the absence of glucose and other carbohydrates or stationary growth phase conditions signaled unfavorable growth conditions to the cells.

The experiments with glucose analogs might give a first hint to the inhibition mechanism. 2-Deoxy-D-glucose is taken up and phosphorylated, but not metabolized any further by most bacteria. This glucose analog did not inhibit LA conjugation, indicating that it is not glucose itself, but a metabolite of glucose degradation that mediates the observed effect. Methyl-α-D-glucoside may be transported but, depending on the organism studied, it may be phosphorylated (Vadeboncoeur & Trahan 1982) or nonphosphorylated (Brocklehurst et al. 1977). The weak inhibitory effect of methyl-α-D-glucoside on LA conjugation seems to contradict the results observed with 2-deoxy-D-glucose. One possible explanation is a slow degradation of methyl-α-D-glucoside, releasing small amounts of glucose (Brocklehurst et al. 1977).

Nothing is known about the possible physiologic advantage of the inhibition of LA conjugation by glucose for the microorganisms. Moreover, the benefit of CLA synthesis itself is obscure. CLA was proposed to be an intermediate in the biodegradation of LA to stearic acid carried out by the concerted activity of rumen microorganisms (Kelly et al. 1998).

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LITERATURE CITED


