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A *trans*10-18:1 enriched fraction from beef fed a barley grain-based diet induces lipogenic gene expression and reduces viability of HepG2 cells

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ABSTRACT

Beef fat is a natural source of *trans (t)* fatty acids, and is typically enriched with either *t*10-18:1 or *t*11-18:1. Little is known about the bioactivity of individual *t*-18:1 isomers, and the present study compared the effects of *t*9-18:1, *cis* (*c*)9-18:1 and *trans (t)*-18:1 fractions isolated from beef fat enriched with either *t*10-18:1 (HT10) or *t*11-18:1 (HT11). All 18:1 isomers resulted in reduced human liver (HepG2) cell viability relative to control. Both *c*9-18:1 and HT11were the least toxic, *t*9-18:1had dose response increased toxicity, and HT10 had the greatest toxicity ($P < 0.05$). Incorporation of *t*18:1 isomers was 1.8–2.5 fold greater in triacylglycerol (TG) than phospholipids (PL), whereas Δ9 desaturation products were selectively incorporated into PL. Culturing HepG2 cells with *t*9-18:1 and HT10 increased ($P < 0.05$) the Δ9 desaturation index (*c*9–16:1/16:0) compared to other fatty acid treatments. HT10 and *t*9-18:1 also increased expression of lipogenic genes (FAS, SCD1, HMGCR and SREBP2) compared to control ($P < 0.05$), whereas *c*9-18:1 and HT11 did not affect the expression of these genes. Our results suggest effects of HT11 and *c*9-18:1 were similar to BSA control, whereas HT10 and *t*-9 18:1 (i.e. the predominant *trans* fatty acid isomer found in partially hydrogenated vegetable oils) were more cytotoxic and led to greater expression of lipogenic genes.

1. Introduction

Consumption of *trans* (*t*) fatty acids have been associated with numerous adverse effects such as increased plasma LDL-cholesterol/HDL-cholesterol ratio, inflammation, insulin resistance, endothelial dysfunction and oxidative stress [1,2]. The main dietary source of *trans* fatty acids has been partially hydrogenated vegetable oil (PHVO), which can contain up to 60% *trans* fatty acids. The *trans* fatty acids in PHVO are mainly in the form of *trans*-octadecenoic acid (*t*-18:1), representing 90–95% of total *trans* fatty acids in PHVO [3]. Among these, *t*9-18:1 (elaidic acid) and *t*10-18:1 are typically the first and second major isomers, making up on average about 28% and 21% of total *t*-18:1 respectively [4]. Internationally, reduction in PHVO in the food supply has become a priority, and this is to the point where PHVO are no longer generally recognized as safe in the USA, and food manufactures have been given three years to take them out of their products [5]. This leaves ruminant meat and milk as the major source of *t*18:1 in human diets.

In ruminant animals (e.g. sheep, cattle, goats), *t*18:1 isomers are produced by rumen microbes during biohydrogenation of dietary polyunsaturated fatty acids (PUFA), and these can be incorporated into meat and milk [6]. When cattle are fed diets with a high forage to grain ratio, *t*11-18:1 is typically 50–70% of *t*-18:1 isomers, but when a low forage to grain diet is fed, ruminal biohydrogenation pathways shift towards *t*10-18:1 production [7]. The most concentrated *trans* fatty acid in ruminant products is,

Abbreviations: ACC, acetyl-CoA carboxylase; Ag+-SPE, silver ion solid phase extraction; BSA, bovine serum albumin; *c*,, *cis*; FAS, fatty acid synthase; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA reductase; HT10, high-*t*10 fraction; HT11, high-*t*11 fraction; MUFA, monounsaturated fatty acids; PHVO, partially hydrogenated vegetable oils; PL, phospholipid; PUFA, polyunsaturated fatty acids; SCD1, stearoyl-CoA desaturase-1; SFA, saturated fatty acid; SREBP1c, sterol regulatory element-binding protein-1c; SREBP2, sterol regulatory element-binding protein-2; *t*, *trans*; TG, triacylglycerol; TLC, thin layer chromatography

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however, frequently assumed to be *t*11-18:1 (vaccenic acid), and this has made it difficult to interpret their human health effects. In contrast to PHVO, epidemiological studies indicate ruminant *trans* fatty acids are not associated with increased risk for coronary heart disease [8–11], and several animal and cell culture studies have noted positive health effects of *t*11-18:1. Clinical trials, however, have shown *trans* fatty acids from both PHVO and ruminant fats have adverse effects on blood lipids and lipoproteins [12,13].

Studying the effects of *t*18:1 isomers in cell culture is made difficult because some isomers (e.g. t9- and *t*11-18:1) are commercially available, while others (e.g. *t*10-18:1) are not. In order to clarify effects of ruminant products with differing *trans* fatty acid composition, a limited number of animal feeding studies have included dairy products enriched with different *t*-18:1 isomers [14,15]. Changes in the *t*-18:1 profile, however, may have led to confounding effects due to changes in the saturated fatty acid (SFA) and *cis*-monounsaturated fatty acid (*c*-MUFA) contents. To alleviate confounding effects of other fatty acids, we developed silver-ion chromatography techniques to isolate *t*-18:1 fractions and individual *t*-18:1 isomers in quantities sufficient to test their metabolism and bioactivity in cell culture [16]. The objective of the present experiment was to compare the effects of *t*18:1 fractions from beef fat enriched with *t*10-18:1 (HT10) or *t*11-18:1 (HT11) with *t*9-18:1 (the major *t*18:1 isomer in PHVO) and *c*9-18:1 (oleic acid) in liver cell culture (HepG2). We chose liver cells because of their central role in the metabolism of *cis* and *trans* fatty acids including β-oxidation, Δ9 desaturation and lipoprotein secretion [17]. In addition, the adverse effects of *trans* fatty acids from PHVO on blood lipoproteins have been suggested to be mediated in part via modulation of hepatic lipogenic gene expression [18,19]. We hypothesized that beef *t*-18:1 fractions with different *t*-18:1 isomer profiles would have distinct effects on HepG2 cells, specifically in terms of cell viability, lipogenic gene expression, and incorporation of *t*-18:1 isomers into cell triacylglycerol (TG) and phospholipid (PL) fractions.

2. Materials and methods

2.1. Fatty acid treatments

The HT11 and HT10 fractions were isolated from banked backfat samples collected from cattle fed forage (hay) and barley grain-based diets respectively. Diets included supplements rich in 18:2n-6 to increase the content of *t*18:1 fatty acids in beef fat. The distribution of *t*-18:1 isomers of the HT11 and HT10 fractions are presented in Table 1. *Trans*-18:1 fractions were isolated using silver ion (Ag^+) solid phase extraction [16]. Briefly, fat was freeze dried, dissolved in toluene and methylated using 0.5 M sodium methoxide [20]. The resulting fatty acid methyl esters were dissolved in hexane and applied to Discovery® Ag^+-SPE columns (750 mg/6 ml, Supelco, Bellefonte, PA, USA). Saturated fatty acids (SFA) were eluted with 10 ml of hexane and the *t*-18:1 fraction was collected with 10 ml 98:2 hexane: acetone (v/v). The t-18:1 FAME were then saponified to free fatty acids using 0.3 M methanolic potassium hydroxide [21].

Table 1
Composition of *t*-18:1 fractions used for HeG2 culture.

Fatty acid (%)	HT10[a]	HT11[a]
*t*6–*t*8-18:1	7.4	4.0
*t*9-18:1	5.7	4.1
*t*10-18:1	69.3	2.9
*t*11-18:1	7.6	65.4
*t*12-18:1	1.8	6.9
*t*13/*t*14-18:1	3.0	6.8
*t*15-18:1	0.7	5.1
*t*16-18:1	0.4	2.9

[a] HT11 = *t*-18:1 fraction enriched with t11-18:1 from a beef cattle fed a grass-hay based diet. HT10 = *t*-18:1 fraction enriched with t10-18:1 from a beef cattle fed a barely grain based diet.

Individual 18:1 isomers including *t*9-18:1 and *c*9-18:1 were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Individual fatty acids and *t*18:1 fractions were complexed with fatty acid free bovine serum albumin (BSA) at a 4:1 M ratio (4 mM fatty acid: 1 mM BSA) as described by Evans et al. [22]. Fatty acid-BSA complexes were then diluted in cell culture media to provide desired final fatty acid concentrations (100 or 200 μM).

2.2. Cell viability

To test viability, cells were cultured with growth medium containing 5% FBS and supplemented with 100 μM or 200 μM of fatty acids for 96 h. Cell proliferation was assessed using CellTiter-Blue cell viability assay (Promega, Madison, WI). Briefly, HepG2 cells were seeded in 96-well plates (~1000 cells/well) and allowed to attach before 300 μl of medium containing 100 or 200 μM of fatty acid treatment was added. At 0 h, 24 h, 48 h, 72 h and 96 h growth medium including fatty acid treatments was replaced with 100 μl/well serum free medium plus 20 μl/well CellTiter 96® AQueous One Solution reagent (Promega, Madison, WI). After 1.5 h incubation at 37 °C in 6% CO2, the absorbance at 490 nm was recorded using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). The viability results for fatty acid treatments were expressed relative to BSA control.

2.3. Culture conditions for gene expression and fatty acid analyses

Human hepatoma HepG2 cells (ATCC; Rockville, MD, USA) were seeded at a density of 1×10^5 cells per well (9.6 cm^2) in 6-well plates, and cultured at 37 °C in 6% CO2 in a growth medium containing Eagle's minimum essential medium (EMEM; ATCC) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Life Technologies, Burlington, ON, Canada). Cell culture medium was changed every two days, and at 24 h post-confluence, FBS was removed from the medium and cells were treated with 100 μM of the fatty acid-BSA complex for 24 h, and cells were used for fatty acid and gene expression analyses. Control cells were cultured with an equal volume of BSA (vehicle control). Two wells of cells were cultured per treatment per experiment, and the experiment was repeated three times.

2.4. RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted from HepG2 cells using the Aurum total RNA fatty and fibrous tissue kit (Bio-Rad Laboratories, Mississauga, ON, Canada). The RNA concentrations were determined by absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and the RNA purity was evaluated using the 260:280 and 260:230 absorbance ratios to ensure both ratios were between 1.8 and 2.1. Integrity of RNA was confirmed by the presence of intact RNA subunits 28S and 18S using an automated capillary electrophoresis QIAxcel system (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg of RNA using M-MLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) in the presence of random hexamer primers and Ribonuclease Inhibitor (Invitrogen) in a total reaction volume of 20 μl. Real-time PCR analysis was performed using a Stratagene Mx3005P QPCR system (Agilent Technologies) using