Effect of trans-Octadecenoic Acid Positional Isomers on Tumor Necrosis Factor-α Secretion in RAW264.7 Cells

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Abstract: We compared the cytotoxic effects and tumor necrosis factor-α (TNF-α) production induced by 13 trans-octadecenoic acid positional isomers (trans-4-C18:1 to trans-16-C18:1) in RAW264.7 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and enzyme-linked immunosorbent assay, respectively. No significant differences were observed in the cytotoxic effects among the 13 trans-C18:1 positional isomers and control on RAW264.7 cells. TNF-α production significantly decreased by treatment of trans-4-C18:1 as compared to control, but no significant differences in TNF-α production were observed among other trans-C18:1 positional isomers and control. These results suggest that the double bond position in trans-C18:1 may affect TNF-α production in cells.

Key words: trans fatty acid, inflammation, octadecenoic acid isomer, RAW264.7, TNF-α

1 Introduction

trans-Fatty acid (TFA) is a non-conjugated unsaturated fatty acid, interrupted by at least one methylene group with carbon–carbon double bond in the trans configuration¹. In food, TFAs are mainly composed of 80-90% trans-octadecenoic acid (trans-C18:1), which is made up of 13 different types of positional isomers with double-bond positions in the range from Δ4 to Δ16². TFAs are formed via two major reactions, and the distribution of trans-C18:1 positional isomers differs depending on the TFA formation pathway. The first reaction involves partial hydrogenation of vegetable oil; partially hydrogenated vegetable oil (PHVO) contains high percentage of elaidic acid (trans-9-C18:1) and trans-10-C18:1³. The second reaction is biohydrogenation that occurs in the rumen of ruminants such as cows, sheep, and goats. Ruminant fat has high percentage of trans-vaccenic acid (trans-11-C18:1)³-⁴. Several studies have shown that cis-fatty acids such as eicosapentaenoic acid, docosahexaenoic acid, and cis-eicosenoic acid exert several beneficial effects on human health, including anti-inflammatory and anti-obesity functions⁵-⁷. In contrast, TFAs have been reported to exhibit adverse effects on human health. Epidemiological studies have shown that high intake of PHVO is associated with inflammation, diabetes, and cardiovascular diseases (CVD)⁸-⁹. The relationship between these disorders and TFAs can be explained by the similarity in the biological behavior of TFAs and saturated fatty acids (SFAs) because both have similar physical properties. For instance, high intake of TFAs increases low-density lipoprotein (LDL) and decreases high-density lipoprotein (HDL) concentrations in the blood, similarly to SFAs⁸⁻⁻⁹. This is a major cause of cholesterol accumulation and inflammation in the artery wall that consequently leads to CVD⁸⁻⁻⁹. Furthermore, in comparison with SFAs, TFAs are known to induce a higher increase in the ratio of LDL/HDL. Thus, TFAs are more severe risk factors for CVD than SFAs⁹⁻⁻¹¹. However, the underlying pathological mechanisms are largely unknown.

In addition to CVD, an epidemiological study has suggested the TFA-mediated increases in the risk of diseases such as systemic inflammation, metabolic syndrome, and neurodegenerative disorders¹². Of these diseases, several studies have investigated the relationship between TFAs and inflammatory cytokines. Han et al. evaluated the...
effects of a diet containing hydrogenated fat on cellular immune response and production of inflammatory cytokines in hypercholesterolemic human subjects, and confirmed that the production of interleukin-6 and tumor necrosis factor-α (TNF-α) in margarine diet (6.7% energy from TFAs) group was significantly higher than those of soybean oil diet (0.6% energy from TFAs) group. Mozaffarian et al. also showed that TFA levels in patients with heart disease were strongly associated with systemic inflammatory marker concentrations. While these studies suggested the pro-inflammatory effects of TFA consumption, the mechanisms underlying the disorders associated with TFA intake are still unclear. Recently, Montakah and Dousti investigated the expression of TNF-α gene in RAW264.7 cells treated with trans-9-C18:1 and observed no relationship between TNF-α gene expression and trans-9-C18:1. Hirata et al. revealed the toxic function of TFAs as enhancers of pro-inflammatory signaling and apoptosis induced by doxorubicin. They showed that trans-9-C18:1, trans-11-C18:1, and linoelaidic acid, but not their corresponding cis-isomers, dramatically enhanced the apoptosis of RAW264.7 cells. These are very important findings to elucidate the effects of TFAs on disease development. Although previous studies have reported the relationship between inflammation and trans-9-C18:1 as well as trans-11-C18:1, the influence of other trans-C18:1 positional isomers in inflammation have been little understood. It is also necessary to study TFA positional isomers other than trans-9-C18:1 and trans-11-C18:1 because their levels in food are unjustifiably ignored. For example, the majority of the trans-C18:1 isomers in PHVO comprises trans-10-C18:1 (accounting for 22.7% of total trans-C18:1 isomers), followed by trans-11-C18:1 (19.6%) and trans-9-C18:1 (11.6%). While trans-11-C18:1 (accounting for 36.0% of total trans-C18:1 isomers) was found to be the main constituent of trans-C18:1 in milk fat, followed by trans-16-C18:1 (12.2%). Only a few studies have investigated the effects of TFA positional isomers on inflammatory response, probably owing to the difficulty involved in obtaining them, given their cost and limited number of suppliers.

The aim of the present study was to estimate the influence of trans-C18:1 positional isomers on inflammation. We synthesized all trans-C18:1 positional isomers and evaluated the production of TNF-α in RAW264.7 cells treated with trans-C18:1 positional isomers. This is the first report on the functionality of all trans-C18:1 positional isomers present in food using cell culture systems.

2 Experimental

2.1 Chemicals and materials

All the trans-C18:1 positional isomers used in this study are shown in Fig. 1 (purity: >99%). The trans-isomers (Δ4-16) of C18:1 fatty acid were synthesized according to previous report. These trans-C18:1 positional isomers or oleic acid (cis-9-C18:1) were dissolved in dimethyl sulfoxide (DMSO) and diluted with Dulbecco’s modified Eagle medium (DMEM, Thermo Fisher Scientific, Waltham, MA). Fatty acids were then mixed with bovine serum albumin (BSA) and incubated at 37°C for 24 h. The final concentration of each fatty acid and BSA in medium was 100 μM. These fatty acid-BSA complexes were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and enzyme-linked immunosorbent assay (ELISA). The final concentration of DMSO in the cultures was 0.01%.

2.2 Cell culture and treatments

Mouse macrophage cell line RAW264.7 cells (DS Pharma Biomedical, Osaka, Japan) were maintained in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL strepto-
2.3 Estimation of cytotoxic effect
The cytotoxic effects of each fatty acid were confirmed using the MTT assay. The treated cell culture medium was replaced with DMEM containing 5 mg/mL MTT reagent (Dojindo Laboratories, Kumamoto, Japan), and incubated at 37°C for 3 h. After removed the supernatant, 0.04 mol/L HCl-isopropyl alcohol was added to each well for solubilization of formazan crystals. The optical density of reduced MTT was measured at 550 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA).

2.4 Determination of TNF-α levels in RAW264.7 cells
TNF-α levels in RAW264.7 cells treated with trans-C18:1 positional isomers were determined by ELISA. The supernatants obtained from cells after treatment with fatty acid-BSA complexes were measured the TNF-α levels using TNF the alpha Mouse Uncoated ELISA kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. The levels of TNF-α were analyzed at 450 nm with an iMark Microplate Absorbance Reader (Bio-Rad Laboratories).

2.5 Statistical analysis
Data are presented as means ± SD. Statistical analyses were performed by one-way ANOVA, followed by Tukey-Kramer test to identify significant differences among groups. Differences were considered significant when the p-value is less than 0.05.

3 Results and Discussion
The cytotoxic effects of each trans-C18:1 positional isomer on RAW264.7 cells were estimated by the MTT assay according to the manufacturer’s instructions. None of the 100 μM of trans-C18:1 positional isomers had toxic effects on RAW264.7 cells (Fig. 2). A few studies also have also shown that trans-9-C18:1 and trans-11-C18:1 have no cytotoxic effects against RAW264.7 cells without stimulation. The results of the cytotoxic effects in the present study also show that trans-9-C18:1 and trans-11-C18:1 exerted no cytotoxic effects on RAW264.7 cells without stimulation. However, previous studies have reported the cytotoxic effect of trans-9-C18:1 and trans-11-C18:1 depending on conditions such as high concentration of TFA, cell type, and with/without stimulation. For instance, when RAW264.7 cells were stimulated with 200 μM trans-9-C18:1 in the presence of the DNA-damaging agent doxorubicin, the cell viability was significantly decreased as compared to oleic acid (cis-9-C18:1) by Sarnyai et al. also showed that the viability of RINm5F rat insulinoma cells decreased below 70% after treatment with 500 μM of trans-9-C18:1 and trans-11-C18:1, which had no effects on cell viability at 250 μM. Therefore, further studies are needed to investigate the cytotoxic effects of trans-C18:1 positional isomers other than trans-9-C18:1 and trans-11-C18:1 with/without stimulation, in different of cell types or at high concentrations of more than 1,000 μM.

As shown in Fig. 3, ELISA assay showed no significant difference in TNF-α production between control cells and those treated with different trans-C18:1 positional isomers, except trans-4-C18:1. Interestingly, trans-4-C18:1 significantly decreased the TNF-α production of RAW264.7 cells as compared with control. Therefore, trans-4-C18:1 could exhibit anti-inflammatory effects on RAW264.7 cells, as conjugated-linoleic acid with a trans double bond configuration has been known to exhibit anti-inflammatory properties. However, the present study was operated under no stimulated condition. Therefore, further investigation is needed to estimate the anti-inflammation effect of trans-4-C18:1 in LPS-stimulated RAW264.7 cells.

No significant difference was observed in TNF-α production in the RAW264.7 cells between oleic acid and different
trans-C18:1 positional isomers. The effects of 13 species of trans-C18:1 positional isomers on TNF-α production are not well estimated. Okada et al. found that 100 μM trans-9-C18:1 exerted no effect on TNF-α production of RAW264.7 cells with and without LPS stimulation (LPS) as compared to oleic acid. However, they did not report the data after treatment with 100 μM of trans-9-C18:1 without LPS stimulation. Our results using 100 μM of trans-9-C18:1 were in line with those reported in a previous study. On the contrary, a previous study showed that 1,000 μM trans-9-C18:1 increased the production of TNF-α in RAW264.7 cells with and without LPS stimulation. However, no significant difference was observed in TNF-α levels of various tissues and organs (retroperitoneal, epididymal, and mesenteric adipose tissues, gastrocnemius muscle, and liver) obtained from Swiss male mice fed with hydrogenated vegetable oil (including 4.66% of trans-9-C18:1 in total fatty acids), lard (including 0.09% of trans-9-C18:1 in total fatty acids), soybeans (without trans-9-C18:1) diets or control (without trans-9-C18:1). Thus, we speculate that high concentrations (such as 1,000 μM) of trans-9-C18:1 may increase TNF-α production in RAW264.7 cells.

In conclusion, this is the first study to describe the relationship between 13 trans-C18:1 positional isomers and inflammation in RAW264.7 cells. Further studies on trans-C18:1 positional isomers are warranted to elucidate the properties of trans-C18:1 positional isomers in various diseases, such as systemic inflammation, metabolic syndrome, neurodegenerative disorders and CVD.

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