Comparison of the Effect of *trans* Fatty Acid Isomers on Apolipoprotein A1 and B Secretion in HepG2 Cells

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Abstract: Intake of *trans* fatty acid (TFA) is believed to change the ratio of low-density lipoprotein (LDL) to high density lipoprotein (HDL) cholesterol in blood, which leads to cardiovascular disease. In this study, thirteen types of TFA including monoene type TFA (*trans*-octadecenoic fatty acid isomers, *t*-18:1 isomers), diene type TFA (*t*9,*t*12-18:2), and triene type TFA (*t*-18:3) were added to cultured HepG2 cells to compare the amount of apolipoprotein A1 and B (those relating to levels of HDL and LDL cholesterol in blood, respectively) being secreted. We found that *trans*-5-18:1 increased the secretion of apolipoprotein B relative to oleic acid (*cis*-9-18:1, control). Secretion of apolipoprotein B was also increased by *t*-18:3; however, the amount was not significant compared with that observed in the control. The secretion amount of apolipoprotein B tended to increase with the number of double bonds in TFA among *trans*-9-18:1, *t*9,*t*12-18:2, and *t*-18:3. The secretion amount of apolipoprotein A1 after TFA treatment was also measured. No significant difference was detected among *t*-18:1 groups; however, *t*-18:3 increased the amount significantly compared to that in the control. These results suggest that the effect of TFA isomers on the ratio of LDL to HDL cholesterol in the blood follows a mechanism different from that in cultured cells.

Key words: apolipoprotein, HepG2 cell, isomer, *trans* fatty acid, secretion

1 INTRODUCTION

*Trans* fatty acid (TFA) exists in food as a source of triacylglycerol, phospholipids, etc. TFA is a geometrical isomer of unsaturated fatty acids possessing at least one double bond in the *trans* configuration. TFA is formed by partial hydrogenation of vegetable oils and biohydrogenation of unsaturated fatty acids by enteric bacteria in the rumen of ruminant animals. Therefore, foods using partially hydrogenated oil (PHO), ruminant milk, and ruminant meat contain TFA. Many epidemiological studies suggest that excessive intake of TFA causes multiple adverse effects that lead to cardiovascular disease (CVD), including dyslipidemia, endothelial dysfunction, and inflammation. Particularly, if more than 2% of daily energy intake is from TFA, the ratio of low-density lipoprotein (LDL) to high density lipoprotein (HDL) cholesterol in blood and the risk for CVD increases drastically. Several epidemiological studies have reported that naturally occurring TFA in ruminant animals is not correlated with CVD. However, the difference between TFAs in PHO and ruminants is ambiguous. PHO, ruminant milk, and ruminant meat contain monoene, diene, and triene types of TFA with the dominant TFA isomer being the monoene type, particularly octadecenoic acid (18:1). Distribution
of the 18:1 TFA positional isomer is nearly identical between PHO and ruminants, and both sources comprise 13 types of isomers \((trans-4-18:1)\) \((t4-18:1)\) – \((t6-18:1)\) \(^{12}\). The predominant \(trans-18:1\) isomer in the ruminants is vaccenic acid \((trans-11-18:1, t11-18:1)\) while the predominant isomer in PHO is elaidic acid \((trans-9-18:1, t9-18:1)\) \(^{12, 13}\). However, ruminant meat also contains \(t9-18:1\) and PHO also contains \(t11-18:1\). The intake amount ratio of ruminant TFA and industrial TFA are not drastically different \(^{12}\).

In the 1990s, Mensink and Katan demonstrated that among 34 women and 25 men, monoene type TFA increased both total and LDL cholesterol and decreased HDL cholesterol \(^{14}\). In the study, the amounts of diene and triene type TFAs were not analyzed. In contrast, Wang \& al. \(^{15}\) recently reported that the intake amount of monoene type TFA is unrelated to cardiovascular disease and total mortality, but that of diene type TFAs strongly relates to mortality \(^{15}\). Though the diene type TFA is not dominant, it is present in foods. Composition of the monoene type (more than 96% was \(t\)-18:1; however, \(t\)-14:1, 15:1, 16:1, 17:1, and 20:1 were also present), diene type, and triene type TFAs in fast food such as hamburgers, pizzas, and French fries have been reported. For example, a hamburger contained 4.08, 0.51, and 0.02% of monoene, diene, and triene type TFAs, respectively \(^{16}\). Vermunt \& al. \(^{17}\) demonstrated in a clinical study that \(trans\) isomers of \(\alpha\)-linolenic acid, which is readily formed during the deodorization of refined vegetable oils, significantly increased the ratio of LDL to HDL cholesterol in plasma compared with low \(trans\) oils \(^{17}\).

Many epidemiological studies focus on the structure of TFA, as it is related to the ratio of LDL to HDL cholesterol in blood or overall mortality. However, the nature of this correlation is unclear and some of the results are in conflict. This is because epidemiological studies prohibit the use of pure TFAs. Several studies examined the effect of \(trans-18:1\) positional isomers on the ratio of LDL to HDL cholesterol in the plasma of hamsters \(^{18, 19}\). Particularly, isomers \(t11-18:1\) (the representative TFA isomer in ruminants) and \(t9-18:1\) (the representative TFA isomer in PHO) were compared. Interestingly, the studies revealed that \(t9-18:1\) did not increase the ratio of LDL to HDL cholesterol in plasma compared with that in the control group, but rather decreased it. The TFA in PHO is believed to increase the ratio of LDL to HDL cholesterol in blood. An \(in vitro\) experiment with HepG2 cells was conducted to compare the effect of TFA isomers \(t8-18:1, t13-18:1\) on the secretion of apolipoprotein B, the primary apolipoprotein of LDL \(^{20}\). The results indicated that the secretion of apolipoprotein B by the addition of \(t8\), \(t9\), \(t11\), \(t12\), and \(t13-18:1\) was not significantly increased compared with that obtained post oleic acid \((cis-9-18:1, c9-18:1)\) treatment. Interestingly, the cells to which \(t10-18:1\) was added showed decreased secretion of apolipoprotein B compared with cells treated with \(c9-18:1\). Thus far, no study has revealed the specific TFA isomer that increases the ratio of LDL to HDL cholesterol in blood. Therefore, we aimed to measure the changes in secretion of apolipoproteins A1, the main apolipoprotein of HDL, and B from HepG2 cells by adding the 13 types of \(t\)-18:1 isomer, diene type TFA, and triene type TFA in order to elucidate the isomers responsible for CVD.

2 EXPERIMENTAL

2.1 Materials
\(c9-18:1\) and \(trans\)-9, \(trans\)-12-linoleic acid \((t9, t12-18:2)\) were obtained from Funakoshi Co., Ltd (Tokyo, Japan); the purity of the fatty acids was greater than 98%. The thirteen kinds of \(t\)-18:1 isomers \((t4-18:1 \sim t16-18:1)\) were produced in-house \(^{21}\). \(trans\) \(\alpha\)-linolenic acid \((t\)-18:3\) was also produced in-house by the isomerization of purified \(\alpha\)-linolenic acid with \(p\)-toluenesulfinate. The \(t\)-18:3 was a mixture of several kinds of triene type TFA isomers. Human hepatoma HepG2 cells and fetal bovine serum (FBS) were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Penicillin–streptomycin was purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fatty acid–free bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). The structures of TFA isomers used in this study are illustrated in Fig. 1.

2.2 Cell culture
HepG2 cells were maintained in DMEM containing 100 units/mL penicillin and 100 \(\mu\)g/mL streptomycin; the cells were supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO\(_2\). At approximately 70–80% confluence, the medium was replaced with a serum-free medium containing 1% BSA (fatty acid-free) for 24 h. To evaluate the effect of \(trans\) fatty acid isomers on apolipoprotein secretion, HepG2 cells were further incubated with either the control medium (1% BSA-DMEM) or experimental medium (1% BSA-DMEM with fatty acids (150 \(\mu\)M) for 24 h, according to the previous analytical condition we reported \(^{22}\). The fatty acid–BSA complex was prepared as described by Van Harken \& al. \(^{22}\). The cellular protein concentration was determined using the bicinchoninic acid method \(^{24}\). To measure cytotoxicity, water-soluble disulfonated tetrazolium salt synthetic activity \(^{25}\) was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

2.3 Measurement of apolipoprotein A1 and B secretion from cells
Apolipoprotein A1 and B levels in the culture medium were quantitated using the Total Human Apolipoprotein A1 ELISA Assay Kit (AlerCHEK, Inc., Springvale, ME, U.S.A.) and Total Human Apolipoprotein B ELISA Assay Kit (AlerCHEK, Inc., Springvale, ME, U.S.A.).
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2.4 Statistical analysis
All values are expressed as means ± SE. The data were analyzed using a one-way ANOVA test, and all differences were evaluated using the Tukey–Kramer post-hoc test (Kaleida Graph, Synergy Software, Reading, PA). Differences were considered statistically significant at \( p < 0.05 \).

3 RESULTS
3.1 Viability of HepG2 cells
The effects of TFAs on cell viability in HepG2 cells were investigated and the results are shown in Fig. 2. c9-18:1 was employed as a control and no cytotoxicity was indicated by any fatty acids. Cellular protein concentrations were not significantly different among groups (data not shown).

3.2 Apolipoprotein A1 and B secretion by the addition of \(-18:1\) isomers
Thirteen types of \(-18:1\) isomers were added to HepG2 cell cultures and the secretion amounts of apolipoprotein A1 and B were measured. c9-18:1 was employed as a control (Fig. 3). There was no significant difference in apolipoprotein A secretion (Fig. 3(A)). In contrast, \( t5-18:1 \) significantly increased apolipoprotein B secretion (Fig. 3(B)). \( t9-18:1 \) and \( t11-18:1 \) did not significantly change apolipoprotein B secretion compared with that observed in the control.

3.3 Apolipoprotein A1 and B secretion by the addition of monoene, diene, and triene type TFAs
The secretion of apolipoprotein A1 and B from HepG2 cell culture by the addition of two kinds of monoene type \( (t5-18:1 \text{ and } t9-18:1) \), diene type \( (t9,t12-18:2) \), and triene type \( (t-18:3) \) TFAs were compared. c9-18:1 was employed as a control (Fig. 4). The \( t-18:3 \) significantly increased the secretion amount of apolipoprotein A1 compared with that observed in the control (Fig. 4(A)). \( t5-18:1 \) significantly increased the secretion of apolipoprotein B compared with control (Fig. 4(B)). \( t-18:3 \) also increased secretion of apolipoprotein B; however, the difference was not significant. The secretion of apolipoprotein B tended to increase with the number of double bonds in TFA among \( t9-18:1, t9,t12-18:2, \) and \( t-18:3 \).

4 DISCUSSION
The word “Trans Fatty Acid” is a generic name for fatty acids having \( trans \) type double bonds in their structure. The definition of TFA by Codex Alimentarius defines it differently as “Trans fatty acids are all the geometrical isomers of monounsaturated and polysaturated fatty acids having non-conjugated, interrupted by at least one methylene group, carbon-carbon double bonds in the \( trans \) configuration.”26 This definition removes fatty acids having conjugated double bonds in the structure, such as conju-
 gated linoleic acid (CLA), owing to one isomer of CLA, namely trans-10,cis-12-linoleic acid (t10,c12-18:2), which functions to improve fat metabolism in an animal body. Incidentally, the other isomer cis-9, trans-11-linoleic acid (c9,t11-18:2), is not reported to have strong health benefits. This reveals that the position of the double bond in the unsaturated fatty acid is critical for its relevance to the health of an animal body.

Total TFA consists of many different TFA isomers. For example, the TFA contained in ruminants and PHO mainly consists of three TFA families such as monoene, diene, and triene types TFAs. More than 80% of TFA in ruminants and PHO is monoene of type trans-18:1. There are thirteen potential positional isomers of trans-18:1. Many researchers have concluded that TFA is bad for health; however, no researcher has revealed which TFA isomer is causally detrimental. From the dramatic effect of the position of the double bond in CLA, it is clear that not all TFA is harmful. Recently, Vahmani et al. examined the effect of trans-18:1 isomers, namely t6-, t9-, t10-, t11-, t13-, t14-, and t15-18:1,
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on triacylglycerol and cholesterol synthesis using HepG2 cells\(^{20}\). They concluded that \(t\)-6, \(t\)-9-, and \(t\)10-18:1 induced lipogenic/cholesterolgenic gene expression. This is a valuable study because the function of respective isomers, having different double bond positions but the same number of carbons and double bonds, was carefully examined. However, the intake of TFA causes a potential risk for coronary disease because TFA has been characterized "to increase the ratio of LDL to HDL ratio in blood"\(^{20}\). This study did not examine the detailed effect on apolipoprotein A1 and B expression, related to HDL cholesterol and LDL cholesterol, respectively. In the current study, we examined the effect of 13 types of \(t\)rans-18:1 isomers on the secretion of apolipoprotein A1 and B from HepG2 cells. Additionally, TFA isomers having two and three double bonds were also evaluated using the same system. None of the fatty acids used in the studies affected the viability of HepG2 cells (Fig. 2). As shown in Fig. 3, \(t\)5-18:1 significantly increased apolipoprotein B secretion. Storkson \textit{et al.} also compared the apolipoprotein B secretion from HepG2 cells by the addition of \(t\)8-18:1 – \(t\)13-18:1\(^{21}\). The addition of \(t\)10-18:1 resulted in the lowest level of secretion while \(t\)13-18:1 resulted in the highest. Our results concur with their findings (Fig. 3(B)). The secretion amount of apolipoprotein A1 was not significantly different among groups; however, the secretion amount of apolipoprotein B significantly increased by the addition of \(t\)5-18:1. Therefore, the \(t\)5-18:1 also would increase the ratio of apolipoprotein B to A1. The change in mRNA expression for apolipoprotein B was measured (data not shown). However, the mRNA did not change significantly compared with that of the control. Data comparing the effect of the number of double bonds on the secretion of apolipoprotein A1 and B are shown in Fig. 4. The result confirms the effect of \(t\)5-18:1 on the secretion of apolipoprotein B from HepG2 cells. Interestingly, \(t\)18:3 increased both apolipoprotein A1 and B compared to the control. \(t\)18:3 was purified using fractionation HPLC, ensuring removal of the \(\alpha\)-linolenic acid precursor. mRNA expression for apolipoprotein A1 and B were measured after diene and triene TFA treatment (data not shown). We found that \(t\)18:3 significantly increased the mRNA level of apolipoprotein A1 compared with that in the control; however, the mRNA expression of apolipoprotein B was not significantly different. The experiment using respective \(t\)18:3 isomers should be conducted to confirm this result. The secretion of apolipoprotein B tended to increase with the number of double bonds in TFA among monoene, diene, and triene types of TFA. As mentioned, several epidemiological studies reported that TFA having two or more double bonds is strongly correlated to the ratio of LDL to HDL cholesterol in blood and on mortality\(^{15}\). Our results support these findings. Studies aimed at revealing the mechanism of TFA action on cholesterol expression are warranted.

5 CONCLUSION

In this study, both \(t\)5-18:1 and \(t\)18:3 increased the secretion amount of apolipoprotein B compared with that in the control. The secretion of apolipoprotein B tended to increase with the number of double bonds in TFA among \(t\)9-18:1, \(t\)9,\(t\)12-18:2, and \(t\)18:3. These results suggest that the effect of TFA on the ratio of LDL to HDL cholesterol varies by the isomer. As these results were obtained from cell culture, animal or human experiments are required to elu-
cidate the effect of individual TFA isomers.

Acknowledgement

This work was supported by Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, Japan (grant number 27009A).

Author contribution

K. N., F. B., and N. G. designed research; K. N. conducted research; K. Y., T. N., H. M., and A. Y. synthesized TFA isomers. K. N. and N. G. wrote paper. K. N. and N. G. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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