Linoleic Acid-Menthyl Ester Reduces the Secretion of Apolipoprotein B100 in HepG2 Cells

Nao Inoue¹, Naomi Yamano¹, Kotaro Sakata¹, Keisuke Arao², Takashi Kobayashi³, Toshihiro Nagao⁴, Yoji Shimada⁴, Koji Nagao¹ and Teruyoshi Yanagita¹

¹ Department of Applied Biochemistry and Food Science, Saga University (Saga 840-8502, JAPAN)
² Department of Health and Nutrition Sciences, Nishikyushu University (Kanzaki 842-8585, JAPAN)
³ Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University (Sakyo-ku, Kyoto 606-8502, JAPAN)
⁴ Biomaterials and Commodity Chemicals Research Division, Osaka Municipal Technical Research Institute (Osaka 536-8553, JAPAN)

Abstract: The effect of linoleic acid-menthyl ester (LAME) on lipid metabolism were assessed in HepG2 cells. It is well known that high level of apolipoprotein (apo) B100 in the serum is risk for atherosclerosis. Although linoleic acid (LA) treatment and LA plus l-menthol treatment increased apo B100 secretion, LAME treatment significantly decreased apo B100 secretion in HepG2 cells compared with control medium. The hypolipidemic effect of LAME was attributable to the suppression of triglyceride synthesis in HepG2 cells. It is also known that the risk of coronary heart disease is negatively related to the concentration of serum apo A-1. In the present study, LAME treatment increased apo A-1 secretion as compared with LA treatment in HepG2 cells. These results suggest that mentyl-esterification of fatty acids may be beneficial in anti-atherogenic dietary therapy.

Key words: linoleic acid-menthyl ester, apolipoprotein B100, apolipoprotein A-1, HepG2 cells

1 INTRODUCTION

Lifestyle-related diseases, such as obesity, hyperlipidemia, atherosclerosis, type 2 diabetes, and hypertension, are widespread and increasingly prevalent in industrialized countries. Accompanied by the rapid increase in the number of elderly people, this becomes a medical and a socioeconomic issue. A clustering of metabolic disorders in an individual, defined as metabolic syndrome, is known to increase cardiovascular morbidity and mortality¹. Although the pathogenesis of metabolic syndrome is complicated and precise details of the underlying mechanisms are not known, dyslipidemia is now proposed as a feature of the metabolic syndrome along with the insulin resistance. Many studies suggested that the quality of dietary fats may be an important modulator in terms of the risks associated with this syndrome². In addition, differential effects have arisen with respect to individual fatty acids. For example, conjugated linoleic acid (CLA), which refers to a mixture of positional and geometric isomers of linoleic acid (LA) with conjugated double bonds, has attracted considerable attention because of its potentially beneficial biological effects in inhibiting carcinogenesis, attenuating atherosclerosis, alleviating diabetes, and reducing body fat in animal models and humans³⁴.

Linoleic acid-menthyl ester (LAME) is a conjugate of LA and l-menthol. Menthol is a cyclic terpene alcohol that has been used as traditional herbal medicines and cooling agents in cosmetic products⁵⁶. Menthol also shows various pharmacological activities including antimicrobial, anti-inflammatory, analgesic, and central nervous system excitation effects⁷-¹⁰. Previous reports indicated that fatty acid-ascorbyl ester, conjugate of fatty acids and ascorbic acid, find attractive applications as an antioxidant in cosmetics, pharmaceuticals and food ingredients¹¹-¹⁴. Additionally, there are studies showing that ascorbyl-palmitate is much more potent than ascorbic acid as a physiologically active compound in vivo and in vitro¹⁴,¹⁵. Therefore, evaluating the physiological functions of synthetic molecules, such as complex of fatty acids and bioactive compounds, would be of great interest. Since we hypothesized that fatty acid-menthol ester could serve as an effective carrier of l-menthol into active domain of lipid metabolism and would reveal some pharmacological activity, in the present study, we evaluated the effect of LAME on lipid metabolism in HepG2 cells.
HepG2 cells.

2 MATERIALS AND METHOD

2.1 Materials
LA, CLA, and l-menthol were obtained from The Nisshin OilliO group (Yokosuka, Japan). LAME was made from LA and l-menthol with Candida rugosa lipase-catalyzed selective esterification. Human hepatoma HepG2 cells and fetal bovine serum (FBS) were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, 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).

2.2 Cell culture
HepG2 cells were maintained in DMEM containing 100 units/mL penicillin and 100 μg/mL streptomycin, and supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO2. At approximately 70-80% confluence, the medium was replaced by a serum-free medium containing 1% BSA (fatty acid-free) for 24 h.

For the evaluation of the effect of LAME on secretion and mRNA expression of apolipoproteins, HepG2 cells were further incubated with either the control medium (1% BSA-DMEM) or experimental media (1% BSA-DMEM with LA (25-150 μM), LAME (25-150 μM), CLA (150 μM), or LA+l-menthol (150 μM)) for 24 h. At the end of experiment, the media were harvested to measure apolipoprotein levels, and the cells were used for the determination of cellular protein and mRNA levels.

For the evaluation of the effect of LAME on cellular triglyceride (TG) synthesis, HepG2 cells were further incubated with either the control medium (1% BSA-DMEM) or experimental media (1% BSA-DMEM with LA (25-150 μM), LAME (25-150 μM), CLA (150 μM), or LA+l-menthol (150 μM)) for 48 h. Before 3 h of cell collection, 9.6 kBq [1-14C] oleate (American Radiolabeled Chemicals, St. Louis, MO, USA) was added to the medium. After incubation, cells were washed once and collected in 2 mL of PBS with using a rubber policeman.

Each experimental fatty acid-BSA complex was prepared as described by Van Harken et al. The cellular protein concentration was determined using the method of Lowry et al. or the bicinchoninic acid method. To measure cytotoxicity, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) activity was determined using a MTT Cell Growth Kit (CHEMICON International, Inc., Temecula, CA, U.S.A.).

2.3 Measurement of apolipoprotein secretion
Apo B100 and apo A-1 levels in the culture media were quantitated using the ApoB Microwell ELISA Assay Kit (AlerCHEK) and ApoA-1 Microwell ELISA Assay Kit (AlerCHEK).

2.4 Measurement of cellular TG synthesis
Cells were thawed and homogenized with a sonicator (Sonifier 250TM, Branson Ultrasonic Co., CT, USA) before analysis. Total lipids in the cells were extracted and purified by the method of Bligh and Dyer, and measured by liquid scintillation counter (Wallac System 1410, Pharmacia, Uppsala, Sweden). The lipids were fractionated using thin-layer chromatography (TLC) in a solvent mixture of petroleum ether: diethyl ether: acetate (82:18:1, v/v). After separation with TLC, radioactivity in the TG fraction was measured with a bio-imaging analyzer (LAS1000, Fuji Photo Film, Kanagawa, Japan).

2.5 Analysis of mRNA expression
Total RNA was extracted from cells, using a RNeasy Micro Kit (Qiagen, Tokyo, Japan). A TaqMan Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan); Assay-on-Demand, Gene Expression Products (Hs00181142_m1 for apo B100; Hs00163641_m1 for apo A-1, Hs99999901_s1 for 18S RNA, Applied Biosystems) were used for the quantitative real-time RT-PCR analysis of apo B100, apo A-1, and 18S RNA in HepG2 cells. The amplification was performed with a real-time PCR system (ABI Prism 7000 Sequence Detection System; Applied Biosystems). Results were quantified with a comparative method and were expressed as a relative value after normalization to the 18S RNA expression.

2.6 Statistical analysis
Each value is presented as means ± standard error. Data were analyzed by one-way analysis of variance, and all differences were examined with Tukey-Kramer or Student’s t test (Super ANOVA, Abacus Concepts, Berkeley, CA). P<0.05 was considered statistically significant.

3 RESULTS

3.1 Effect of LAME on apo B100 secretion in HepG2 cells
Although LA treatment and LA plus l-menthol treatment increased apo B100 secretion, LAME treatment significantly decreased apoB100 secretion in HepG2 cells compared with control medium. The lowering effect of LAME was similar to that of CLA, known as a strong hypolipidemic fatty acid, in HepG2 cells (Fig. 1).

3.2 Effect of LAME on cellular TG synthesis
Figure 2 indicates the incorporation of [1-14C] oleate into the cellular TG fraction in HepG2 cells after 3 h incubation. LA treatment tended to increase the incorporation of [1-
LA-Menthyl Ester Reduces ApoB100 Secretion

14C] oleate into cellular TG, which meant enhanced TG synthesis, but the increase was significantly reduced by LAME as compared with the other groups.

3.3 Effect of various concentration of LAME on apo B100 secretion in HepG2 cells

We investigated the effect of various concentrations (25-100 μM) of LA or LAME on the secretion of apo B100 in HepG2 cells (Fig. 3). LAME supplementation significantly decreased apo B100 secretion compared with the control medium (at all range: 25-100 μM) and with the same concentration of LA group (at 25 and 100 μM).

3.4 Effect of LAME on apo A-1 secretion in HepG2 cells

As shown in Fig. 4, LA significantly decreased apo A-1 secretion as compared with the control group. However, LAME normalized apo A-1 secretion to control level in HepG2 cells.

3.5 Effect of LAME on mRNA expression of apolipoproteins in HepG2 cells

To investigate the regulation of apo B100 and apo A-1 production, we analyzed the mRNA expression of apolipoproteins in HepG2 cells. As shown in Fig. 5, mRNA expressions of apoB100 and apoA-1 were not affected by LAME.
DISCUSSION

It is known that liver is the pivotal organ concerned with lipid metabolism and apo B100-containing lipoproteins assembly and secretion\textsuperscript{22}). Overproduction of apo B100-containing lipoprotein in the liver results in hyperlipidemia. Furthermore, higher concentrations of serum cholesterol, TG, and apo B100 are major risk factors on coronary heart disease (CHD) and atherosclerosis\textsuperscript{23,24}). Human hepatoma HepG2 cells are the most suitable and accessible human-derived cells and retain many of the biochemical functions of human liver parenchymal cells. The viabilities of HepG2 cells, as assessed by MTT assay, after 24 h incubation with experimental media were not significantly different among all groups (data not shown). Therefore, we conclude that LAME at the concentrations used in this study (25-150 \textmu M) is not cytotoxic to HepG2 cells.

Figure 1 shows the apo B100 accumulation in the medium. Although LA treatment increased apo B100 secretion, LAME markedly decreased apo B100 secretion compared with control medium. The lowering effect was similar to that of CLA, known as a strong hypolipidemic fatty acid, in HepG2 cells. Given the result in this study that treatment with LA plus free form of \textgamma-menthol increased apo B100 secretion, LAME may exert hypolipidemic property as an intact ester molecule. Apo B100, a 556-kDa hydrophobic protein, is synthesized in the endoplasmic reticulum\textsuperscript{22}). Following synthesis, apo B100 is stabilized by binding with lipids, and then assembled and secreted as apo B100 containing lipoprotein such as very low density lipoprotein\textsuperscript{22}). Because TG availability is a major factor in the regulation of apo B100 secretion\textsuperscript{25)}, the present study examined the effect of LAME on cellular TG synthesis in HepG2 cells. Figure 2 indicates the incorporation of [1-\textsuperscript{14}C] oleate into the cellular TG fraction in HepG2 cells after 3 h incubation. LA treatments tended to increase [1-\textsuperscript{14}C] oleate incorporation into cellular TG, which meant enhanced TG synthesis, but the increase was significantly reduced by LAME as compared with control medium. This result is consistent with our previous report showing that conjugated fatty acids reduced apo B100 secretion through the suppression of TG synthesis in HepG2 cells\textsuperscript{26,27}). Hence we consider that the reduction of apo B100 secretion by LAME is at least in part attributable to the suppression of cellular TG synthesis in HepG2 cells. Figure 3 shows the effect of various concentrations (25-100 \textmu M) of LA or LAME on the secretion of apo B100 in HepG2 cells. LAME treatment significantly decreased apo B100 secretion compared with the control medium (at all range: 25-100 \textmu M) and with the same concentration of LA group (at 25 and 100 \textmu M). Thus, the lowest effective concentration of LAME would be 25 \textmu M for the present.

Epidemiologic studies have established that the risk of CHD is negatively related to the concentration of high density lipoprotein (HDL) cholesterol and their major apolipoprotein, apo A-1\textsuperscript{24,28}). As shown in figure 4, LA treatment significantly decreased apo A-1 secretion as compared with the control medium. However, LAME (mentyl-esterificated LA) treatment normalized apoA-1 secretion to control level in HepG2 cells. This result suggests that LAME has the elevative effect on plasma HDL.

To further investigate the regulation of apo B100 and apo A-1 secretion, we analyzed the effect of LAME on mRNA expression in HepG2 cells. As shown in figure 5, mRNA expressions of apo B100 and apo A-1 were not affected by LAME. Accordingly, secretions of apo B100 and apo A-1 by LAME might be regulated posttranscriptionally.

In conclusion, the present study demonstrated that LAME reduces apo B100 secretion through the suppression of TG synthesis in HepG2 cells. Additionally, LAME treatment increased apo A-1 secretion as compared with LA treatment in HepG2 cells. Therefore, mentyl-esterification of fatty acids is expected to be a useful procedure for providing anti-atherogenic components. There is, however, no information on the bioavailability of LAME after oral ingestion. Metabolic fate and nutraceutical effect of LAME in vivo must be elucidated in future study.

ACKNOWLEDGMENTS

We thank Kimiko Yasuda for technical assistance and thank The Nisshin OilliO group for providing experimental samples. This work was supported by research grants from the 21st Century COE Program of the Japanese Ministry of Education, Culture, Sports, Science and Technology.
LA-Menthyl Ester Reduces ApoB100 Secretion

References


