Implitapide, a Microsomal Triglyceride Transfer Protein Inhibitor, Reduces Progression of Atherosclerosis in Apolipoprotein E Knockout Mice Fed a Western-Type Diet: Involvement of the Inhibition of Postprandial Triglyceride Elevation

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Microsomal triglyceride transfer protein (MTP) is essential for the synthesis of both chylomicron in the intestine and very low-density lipoprotein in the liver. An MTP inhibitor, (2S)-2-cyclopentyl-2-[4-[(2,4-dimethyl-9H-pyrido[2,3-b]indol-9-yl)methyl][phenyl]-N-[(1S)-2-hydroxy-1-phenylethyl]ethanamide (implitapide), has been shown to suppress atherosclerosis in apolipoprotein E knockout (apoE KO) mice. To elucidate the antiatherosclerotic mechanisms of implitapide in the mice, we examined the effects on plasma lipid levels, triglyceride (TG) elevation after oral fat loading, and development of atherosclerosis in apoE KO mice fed a Western-type diet. Implitapide at a dosage of approximately 3.2 mg/kg/day significantly reduced both total cholesterol and TG levels during the 8-week treatment period. In addition, implitapide significantly inhibited the increase in plasma TG levels after oral olive oil loading tests conducted after 4 weeks of treatment. After the treatment, implitapide significantly suppressed the atherosclerotic lesion area by 83% compared with a control group. These results provide direct evidence that the antiatherosclerotic effects of implitapide in apoE KO mice are associated with the inhibition of postprandial TG elevation, in addition to the reduction of both plasma total cholesterol and TG levels.

Key words microsomal triglyceride transfer protein; implitapide; atherosclerosis

Patients with atherogenic dyslipidemia are at high risk for coronary heart disease (CHD).5 Guidelines from the National Cholesterol Education Program recommend reduction of low-density lipoprotein (LDL) cholesterol as the primary goal in cardiovascular risk reduction therapy.5 According to the guidelines, statins are widely used for LDL cholesterol-lowering therapy. Recently, meta-analyses of prospective studies indicated that increased triglyceride (TG) levels were also an independent risk factor for CHD.5 Furthermore, abnormal postprandial accumulations of remnants from TG-rich lipoproteins, such as chylomicron and very low-density lipoprotein (VLDL), have been shown to be linked to CHD regardless of fasting TG levels.5 Fibrates are often used for TG-lowering therapy and have lowering effects on postprandial TG levels as well as fasting TG levels.5 However, there are no potent lipid-lowering agents that reduce both cholesterol and TG levels.

Microsomal TG transfer protein (MTP) plays an important role in the assembly of apolipoprotein B (apoB)-containing lipoproteins such as VLDL in the liver and chylomicron in the intestine.5,7 MTP inhibitors have been shown to reduce both total cholesterol and TG levels not only in animal experiments8,9 but also in clinical studies.9 In addition, MTP inhibitors reduced the plasma TG rise after oral fat loading in rodents.8,9 Therefore MTP inhibitors should be useful for prevention of atherosclerotic diseases in patients with combined hyperlipidemia, such as type IIb and III hyperlipidemia.

Apolipoprotein E knockout (apoE KO) mice are widely used as an animal model of atherosclerosis. When fed a Western-type diet (WD), these mice develop severe hyperlipidemia and atherosclerosis.10,11 In this mouse model, an MTP inhibitor, (2S)-2-cyclopentyl-2-[4-[(2,4-dimethyl-9H-pyrido[2,3-b]indol-9-yl)methyl][phenyl]-N-[(1S)-2-hydroxy-1-phenylethyl]ethanamide (implitapide), has been shown to reduce progression of atherosclerosis.12 To elucidate the antiatherosclerotic mechanisms of implitapide in the mouse model of atherosclerosis, we examined the effects on plasma lipid levels, TG elevation after oral fat loading, and development of atherosclerotic lesions in apoE KO mice fed a WD.

MATERIALS AND METHODS

Materials Implitapide was synthesized at Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Sodium pentobarbital (Nembutal) was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). TG E Test Wako, Cholesterol E Test Wako, and oil red O were purchased from Wako Pure Chemical (Osaka, Japan). All other reagents were purchased from Nacalai Tesque (Osaka, Japan).

Animals and Diets Homozygous apoE KO mice were bred from breeding pairs obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.). C57BL/6J mice were purchased from SLC Ltd. (Shizuoka, Japan). Animals were housed in a temperature-controlled facility with a 12-h light/dark cycle and allowed ad libitum access to water. The WD, containing 21% (w/w) fat and 0.15% (w/w) cholesterol, and chow diet (CD), containing 5% (w/w) fat, were prepared by Oriental Yeast (Osaka, Japan). All animal experimental procedures were performed in accordance with guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical Co., Ltd.

Experimental Protocol Male apoE KO mice aged 6 weeks were fed either the CD or the WD. At age 7 weeks, apoE KO mice fed the WD were divided into two groups with similar mean body weight: apoE KO mice fed the WD and apoE KO mice fed the WD containing implitapide (WI). Age-matched C57BL/6J mice fed the CD were used as a naive control (C57BL).
Implitapide concentrations (14—22 ppm) in the diet were adjusted once a week to ensure dosage consumption of approximately 3.2 mg/kg/d. This dosage was based on a preliminary study, which showed that implitapide at a dosage of approximately 3.2 mg/kg/d, but not at a dosage of approximately 1 mg/kg/d, significantly reduced both total cholesterol and TG levels in apoE KO mice fed a WD. Body weight and average food consumption for 3 d were monitored weekly. Before and at 4 and 8 weeks of treatment, blood was collected for measurements of plasma lipid levels. At the 4th week, an oral fat-loading test was performed. At the 5th week of treatment, feces were collected for determination of fecal fat. At the end of 8 weeks of treatment, mice were euthanized for analysis of atherosclerotic lesions.

**Plasma Lipid Measurement and Lipoprotein Fractionation** Blood was collected from the retroorbital venous plexus. Plasma was separated, and total cholesterol and TG levels were enzymatically measured with Cholesterol E Test Wako and TG E Test Wako, respectively. For lipoprotein fractionation, plasma samples of each group were pooled and electrophoresed on 1% agarose gel (Titan Gels, Helena Laboratories, Saitama, Japan). The cholesterol in lipoprotein fractions was stained with the CHOL/TRIG Combo system (Wako Pure Chemical). The cholesterol in lipoprotein fractions was stained with the CHOL/TRIG Combo system and scanned by a densitometer (EDC, Helena Laboratories).

**Oral Fat-Loading Test** To assess the postprandial increase in TG-rich lipoproteins, oral fat-loading tests were performed. Olive oil was chosen for the loading tests because of its safety and adequate fluidity. Mice fasted for 16 h were orally administered 10 ml/kg of olive oil emulsion, containing 30% olive oil, 5% Tween-20, and 65% methylcellulose solution (0.5% w/v). Blood samples were collected before (0 h), and 2 and 4 h after oral fat loading. Plasma was separated and TG levels were measured as an index of remnant-like particles, since changes in TG levels after oral fat loading were reported to be correlated with changes in remnant-like particles derived from TG-rich lipoproteins. The area under the curve of plasma TG levels above the initial level (AUC\(\text{TG}_{0-4h}\)) was calculated.

**Determination of Fat in Feces** The feces were collected for 3 d, dried, and pulverized in a mill. The amount of fat in feces was analyzed using the method of van de Kamer et al. Briefly, fatty acids and fat were extracted with petroleum ether from an acidic, alcoholic solution containing the sample of feces, and the amount of extract was measured gravimetrically. The amount of fat ingested was determined by multiplying food intake by the percentage of food as fat. The percentage of fat excreted was calculated by dividing the amount of fat ingested by the amount of fat excreted under sodium pentobarbital anesthesia, the thoracic aorta and heart were perfusion-fixed with 4% paraformaldehyde (pH 7.4). For the analysis of en face lipid staining, the thoracic aorta was opened longitudinally and pinned out on a black corkboard. Fatty streak lesions were stained with oil red O (Wako Pure Chemical). En face images of the aorta were taken with a digital camera (Nikon, Tokyo, Japan). Quantification of the images was performed using Mac Scope software (Mitani, Fukui, Japan). Aortic lesion areas were calculated by dividing oil red O-stained areas by the total area. For observation of the aortic root, the heart was embedded in OCT compound (Sakura, Tokyo, Japan) or paraffin. Cryostat sections (6 μm) were stained with hematoxylin and oil red O. Serial 5-μm thick sections of the paraffin-embedded heart were stained with hematoxylin and eosin or orcein to improve the visibility of elastic tissues. Sections were observed under a light microscope (Nikon, Tokyo, Japan).

**Statistical Analysis** Data are expressed as mean±S.E.M. if not otherwise stated. Statistical analysis of mean differences was performed with Student's t-test and Dunnnett's multiple comparison test. Values of \(p<0.05\) were regarded as statistically significant.

**RESULTS**

**Body Weight and Food Consumption** Body weight changes and food consumption during the feeding period are shown in Table 1. The initial body weight of the CD group was significantly lower than that of the C57BL group, but there was no significant difference in body weight gain. Food consumption during the feeding period was significantly higher in the CD group than that in the C57BL group (\(p<0.01\)). The body weight gain of the WD group during the feeding period was similar to that of the CD group, in spite of significantly lower food consumption (\(p<0.01\)). The WI group had significantly lower final body weight as compared with that of the WD group (\(p<0.01\)), although the WI group showed a tendency toward increased food consumption.

**Plasma Lipid Levels** Both total cholesterol and TG levels were higher in the CD group than in the C57BL group (Fig. 1). These lipid elevations in apoE KO mice were augmented by the WD feeding. Implitapide significantly reduced the plasma lipid levels to nearly or below the CD level at 4 and 8 weeks of treatment (\(p<0.01\)). In the pooled samples of plasma collected after 4 weeks of treatment, VLDL and LDL cholesterol levels were obviously lower in the WI group compared with the WD group (\(p<0.01\)). However, high-density lipoprotein (HDL) cholesterol levels in the WD group and the WI group were almost the same.

**Table 1. Body Weight and Food Consumption in apoE KO Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Body weight (g)</th>
<th>Food consumption (g/mouse/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>C57BL</td>
<td>10</td>
<td>23.1±0.3</td>
<td>32.2±0.6</td>
</tr>
<tr>
<td>CD</td>
<td>8</td>
<td>20.8±0.3*</td>
<td>31.1±0.9</td>
</tr>
<tr>
<td>WD</td>
<td>8</td>
<td>21.0±0.4</td>
<td>30.8±0.8</td>
</tr>
<tr>
<td>WI</td>
<td>8</td>
<td>20.5±0.3</td>
<td>28.5±0.4*</td>
</tr>
</tbody>
</table>

At 7 weeks of age (initial), diets were changed as indicated and were given for 8 weeks. C57BL/6J mice were fed a chow diet (C57BL). ApoE KO mice were fed a chow diet (CD), or a Western-type diet without (WD) or with implitapide approximately 3.2 mg/kg/d (WI). Data are expressed as mean±S.E.M. \(* p<0.01\) compared with C57BL. \(\dagger p<0.01\) compared with CD. \(\dagger\dagger p<0.01\) compared with WD.
An oral fat-loading test was performed after 4 weeks of treatment. After 16-h fasting, baseline TG levels of the WI group were significantly lower than those of the WD group (55±10 mg/dl and 138±14 mg/dl, respectively, p<0.01) (Fig. 3A). Plasma TG levels after oral fat loading were also lowered by implitapide treatment, and the ΔAUC of TG level in the WI group was significantly reduced by 37% compared with that in the WD group (p<0.01) (Fig. 3B).

**Fecal Fat Excretion** At the 5th week of treatment, excretion of fecal fat in the CD group and the WD group was 4% and 2% of ingested fat, respectively (Fig. 4). Excretion of fecal fat in the WI group was markedly higher (15% of ingested fat) than that in the WD group.

**Atherosclerosis** Typical lipid-stained sections of the aortic root are shown in Fig. 5. There were no lipid-stained lesions in the C57BL group (Fig. 5A). The CD group demonstrated little lipid-stained areas in the entire neointima (Fig. 5C, E). Implitapide markedly suppressed lipid-stained lesions in the mice fed the WD (Figs. 5D, F). As shown in Figs. 6A and C, the aortic root obtained from the WD group demonstrated gross intimal thickening and cholesterol crystal accumulation. These alterations of such lesions were mitigated in the WI group (Figs. 6B, D). Figure 7 shows the lipid-stained lesion areas at the en face surface thoracic aorta. The lesion area of the WD group was markedly larger than that of the CD group (p<0.01). Implitapide significantly decreased lesion area by 83% compared with that of the WD group (p<0.01).

**DISCUSSION**

The present study demonstrated that implitapide signifi-
Fig. 5. Photomicrographs of the Aortic Root in apoE KO Mice after 8 Weeks of Treatment

Cryostat sections from C57BL/6J mice fed a CD (C57BL; A), apoE KO mice fed a CD (B), and apoE KO mice fed a WD without (WD; C and E) or with implitapide at a dosage of approximately 3.2 mg/kg/d (WI; D and F) were stained with hematoxylin and oil red O (original magnifications: ×100 in A—D; ×400 in E and F). Horizontal bars represent 100 μm.

Fig. 6. Photomicrographs of the Aortic Root in apoE KO Mice after 8 Weeks of Treatment

Sections of the paraffin-embedded heart from apoE KO mice fed a WD without (WD; A and C) or with implitapide at a dosage of approximately 3.2 mg/kg/d (WI; B and D) were stained with hematoxylin and eosin (A and B) or orcein (C and D) (original magnifications: ×100). Horizontal bars represent 100 μm.
Implitapide was shown to suppress MTP activity using a recombinant human form complexed with protein disulfide isomerase (IC50 = 10 nM) and inhibit secretion of apoB-containing VLDL-like lipoproteins from a human hepatoma cell (HepG2) with an IC50 value of 1.1 nM. Our study demonstrated that implitapide at a dosage of approximately 3.2 mg/kg/d significantly reduced plasma total cholesterol and TG levels in apoE KO mice. Thus it was confirmed that this dose of implitapide is sufficient for the suppression of atherosclerosis in apoE KO mice.

MTP inhibitors have been shown to reduce both plasma total cholesterol and TG levels in normolipidemic mice, rats, and dogs. Similar effects were also observed in Watanabe-heritable hyperlipidemic rabbits, a model of human homozygous familial hypercholesterolemia. Our study demonstrated that implitapide at a dosage of approximately 3.2 mg/kg/d significantly reduced the lipids-stained aortic lesions by 83% in apoE KO mice. Thus it was confirmed that this dose of implitapide is sufficient for the suppression of atherosclerosis in apoE KO mice.

In conclusion, this study provides direct evidence that the antiatherosclerotic effects of implitapide in apoE KO mice is associated with the inhibition of postprandial TG elevation, in addition to the reduction of both plasma total cholesterol and TG levels. Inhibition of MTP may be a promising strategy for the prevention of atherosclerotic diseases in patients with combined hyperlipidemia, such as type IIb and III hyperlipidemia.

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