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# Pactimibe stabilizes atherosclerotic plaque through macrophage acyl-CoA: cholesterol acyltransferase inhibition in WHHL rabbits

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**Abstract**

Novel acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitor pactimibe was administered as the sulfate salt form to 3-month-old homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits at doses of 0, 10, or 30 mg/kg for 32 weeks. Pactimibe (10 and 30 mg/kg) tended to reduce intimal thickening in thoracic aortic lesions (294±39 and 276±32 μm, respectively, versus 313±37 μm control), histopathological examination revealing significantly increased smooth muscle cell area (12.0±0.9% and 12.3±0.5%, $P<0.05$, respectively, versus 9.7±0.8% control), significantly increased collagen fiber area (20.5±1.2% and 31.0±1.3%, $P<0.05$, respectively, versus 16.2±1.0% control), and tended to reduce macrophage infiltration (6.0±1.1% and 4.6±1.0%, respectively, versus 7.0±1.3% control). Pactimibe dose-dependently reduced cholesteryl ester content in thoracic and abdominal aortic lesions, and reduced free cholesterol content in the aorta versus control.

Although pactimibe did not alter serum cholesterol levels in WHHL rabbits, it stabilized vulnerable plaque characterized with reduced cholesteryl ester content, enriched collagen fibers and increased smooth muscle cells, indicating potential as a treatment strategy for coronary heart disease.


*Keywords:* Atherosclerosis; Plaque; Collagen; ACAT inhibitor

## 1. Introduction

Recent clinical studies have shown that lipid lowering with 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, hereafter referred to as statins, can reduce coronary events and mortality rates (Sacks et al., 1996; Scandinavian Simvastatin Survival Study (4S) Group, 1994; Shepherd et al., 1995). Davies et al. (1993), Davies (1996) have also distinguished several features of ruptured plaques. Lesions that cause fatal coronary thrombus are typically composed of large lipid cores underlying thin fibrous caps poor in smooth muscle cells and collagen. Lipid-lowering therapy by diet or statin reduced lipid core and macrophage accumulation, thus causing fewer rupture-prone lesions (Aikawa et al., 1998; Fukumoto et al., 2001). However, despite the impressive results obtained with these agents, the majority of cardiovascular events are not prevented by treatment with statins. Although contribution of increased low density lipoprotein (LDL) cholesterol as a proatherogenic factor is documented, most coronary artery disease patients develop acute coronary events despite normal plasma LDL cholesterol levels. This clearly indicates that vascular inflammation enough to cause plaque rupture is present independent of plasma LDL cholesterol values. Recent clinical observations to measure vascular markers such as high sensitive C-reactive protein support this concept (Ridker, 2005). Thus, additional therapies to directly stabilize vulnerable plaque independent of the plasma lipid profile are necessary in order to further reduce the coronary event rate.

Acyl coenzyme A:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is an intracellular enzyme that catalyzes the formation of cholesteryl esters from cholesterol and fatty acyl-coenzyme A (Chang et al., 1997). Arterial ACAT activity could account for cholesteryl ester accumulation in macrophage foam cell formation. Therefore, administration of an ACAT inhibitor with systemic bioavailability could be expected to directly prevent

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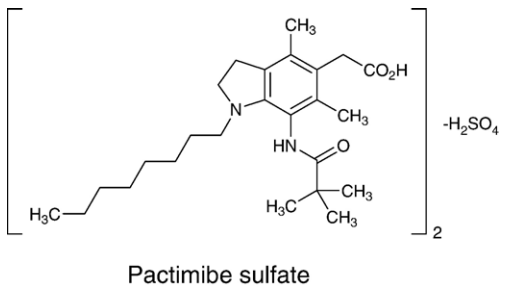
foam cell formation in the arterial wall (Sliskovic and White, 1991). There have been several studies reporting anti-atherosclerotic efficacy by ACAT inhibitors in hyperlipidemic models (Asami et al., 1998; Bocan et al., 1991; Nicolosi et al., 1998). The ability of ACAT inhibitors to limit atherogenesis appears to be beyond lower plasma cholesterol levels.

We tested the hypothesis that an ACAT inhibitor stabilizes atherosclerotic plaque by reducing the accumulation of cholesteryl ester that can alter plaque vulnerability, thereby reinforcing the ability of the plaque to resist rupture without significant cholesterol-lowering effect in Watanabe heritable hyperlipidemic (WHHL) rabbits. We herein report the changes produced by administration of a novel ACAT inhibitor, pactimibe sulfate, [7-(2,2-dimethylpropanamido)-4,6-dimethyl-1-octylindolin-5-yl]acetic acid hemisulfate (formerly named CS-505, Fig. 1), in accumulation of macrophages and smooth muscle cells, cholesterol content, and the amount and distribution of the collagenous extracellular matrix in atherosclerotic lesions in rabbits. These results provide new insight into the potential for cellular mechanisms whereby reduction in lipid content in plaques may produce clinical benefit and support the concept of functional stabilization of features associated with plaque vulnerability.

## 2. Methods

### 2.1. Animals and protocol

A total of thirty homozygous WHHL rabbits (Kitayama Labes Co., Ltd., Ina, Japan) weighing 2.0–2.7 kg at three months old were used in this study. All animals were individually caged in a room controlled for temperature (20–24 °C), humidity (40–70%) and light (6 AM–6 PM) and received 100 g/day of a standard rabbit chow (RC-4, Oriental Yeast Co., Ltd., Tokyo, Japan). Animal experiments were carried out at Nihon Bioresearch Inc. (Gifu, Japan) according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co., Ltd. (Tokyo, Japan). We used male and female rabbits because there are no sex-related differences in atherosclerosis in WHHL rabbits (Shiomi et al., 1992). These rabbits were divided into three groups to make the group serum total cholesterol levels approximately equivalent. The rabbits were given vehicle only ($n=10$, one male and nine females), or 10 mg/kg per day ($n=10$, five males and five females) or 30 mg/kg per day ($n=10$, eight males and two females) of pactimibe in 5% gum arabic suspension in water for 32 weeks.




Pactimibe sulfate

Fig. 1. Chemical structure of pactimibe sulfate.

### 2.2. Tissue preparation

Rabbits were anesthetized with pentobarbital (Nembutal®-sodium solution, Abbott Labs.), given an intravenous injection of heparin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) at 400 U/kg body weight to avoid blood clotting, and sacrificed by exsanguination from the carotid artery. Immediately after laparotomy, they were perfused with saline for 5 min and then with 10% neutral buffered formalin using a perfusion apparatus for 20 min. After perfusion fixation, the aorta and heart were excised. The aortic luminal surface was photocopied to measure the surface lesion area. Cross sections of the aortas were prepared according to the method described previously (Shiomi et al., 1992, 1995, 1990). For preparation of histological specimens, the aorta was divided into five portions as follows: the aortic arch, the proximal and distal portions of the thoracic aorta, and the proximal and distal portions of the abdominal aorta. Finally, the segments that showed macroscopically the most severe lesions in each portion were embedded in paraffin after immersion-fixation, and more than 10 sections, 3 μm in thickness, were cut serially from each segment.

### 2.3. Morphometric methods

The percentage surface lesion area was measured at the aortic arch, the thoracic aorta, and the abdominal aorta using photocopies of the aortas prepared before immersion-fixation according to the method described previously (Tsubamoto et al., 1994). We traced the contours of the lumen and lesion, and then measured morphometrically the surface lumen areas and the surface lesion areas using an imaging analyzer (Quantiment 500+, Leica Cambridge Ltd., Cambridge, UK). The percentage surface lesion area was calculated by dividing the lesion area by the lumenal area.

For the general histopathological examination, histologic cross sections were stained with hematoxylin and eosin as well as with elastica von Gieson.

The average intimal thickening was evaluated using histopathological sections stained with elastica-hematoxylin and eosin (elastica-HE). We measured the lesion area and the length of the external elastic lamina of these sections using a color image analyzer (SPICCA II, Olympus Optical Company Ltd., Tokyo, Japan) equipped with a biological microscope (VANOX, Olympus Optical Company Ltd., Tokyo, Japan) and calculated the average lesional thickening by dividing the lesion area by the length of the external elastic lamina. Data for average intimal thickening of the thoracic and abdominal aortas were estimated as the average of the data for the proximal and distal portions.

Lesional components of the proximal thoracic aortas were quantified according to the method described previously (Shiomi et al., 1995). In brief, serial sections from each segment were quantified immunohistochemically with antibody against human alpha-smooth muscle actin (1A4, DAKO Japan Co., Ltd., Tokyo, Japan) and against rabbit macrophages (RAM 11, DAKO Japan Co., Ltd.), respectively. The sections were also stained with Azan–Mallory's stain. Each specimen was quantitatively evaluated under a color image analyzer (SPICCA II, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a microscope (BX-50,

Table 1
Changes in serum lipid levels in WHHL rabbits

| Treatment group | *n* | 0 wk | 8 wk | 16 wk | 24 wk | 32 wk |
|---|---|---|---|---|---|---|
| Total cholesterol, mg/dl | | | | | | |
| Control | 10 | 944±30 | 867±30 (92±2) | 754±25 (80±2) | 645±23 (69±2) | 611±25 (65±2) |
| Pactimibe 10 mg/kg | 10 | 944±48 | 796±44 (84±2) | 750±44 (79±2) | 653±46 (69±2) | 633±40 (67±2) |
| Pactimibe 30 mg/kg | 10 | 944±33 | 759±24 (81±3) | 706±28 (75±3) | 629±25 (67±2) | 598±29 (64±3) |
| Triglycerides, mg/dl | | | | | | |
| Control | 10 | 111±9 | 141±11 (130±7) | 110±8 (102±9) | 111±11 (102±8) | 111±9 (104±10) |
| Pactimibe 10 mg/kg | 10 | 176±29 | 254±28 (154±10) | 214±30 (128±7)[a] | 235±29 (142±9)[b] | 242±29 (146±8)[b] |
| Pactimibe 30 mg/kg | 10 | 181±25 | 266±25 (155±9) | 236±31 (133±5)[a] | 253±27 (146±6)[b] | 270±31 (154±4)[b] |
| Phospholipids, mg/dl | | | | | | |
| Control | 10 | 459±12 | 463±15 (101±2) | 396±11 (87±1) | 351±9 (77±1) | 347±10 (76±2) |
| Pactimibe 10 mg/kg | 10 | 480±22 | 468±23 (98±1) | 427±20 (89±1) | 384±20 (80±2) | 390±19 (81±2) |
| Pactimibe 30 mg/kg | 10 | 483±18 | 458±13 (95±2) | 414±18 (86±3) | 378±16 (78±2) | 390±17 (81±3) |

Data are expressed as mean±S.E.M. Values in parentheses indicate % of initial values.
[a]$P<0.05$, [b]$P<0.01$ vs. respective control.

Olympus Optical Co. Ltd.). We defined cells with brown reaction product after 1A4 staining as smooth muscle cells, and cells with black product after RAM 11 staining as macrophages. In the sections treated with Azan–Mallory's stain, fibers stained blue were considered to be collagen. The vulnerability index, $[A/(B+C)]$, was calculated from the percent areas of macrophage ($A$), smooth muscle cells ($B$), and collagen ($C$), as described previously with a minor modification (Shiomi et al., 2001a,b).

According to recent literature, complete deficiency of ACAT activity resulted in massive xanthomatosis because of overwhelming free cholesterol deposition in the skin and brain (Fazio et al., 2001; Yagyu et al., 2000). Therefore, we furthermore determined xanthomatosis after pactimibe treatment in WHHL rabbits because WHHL rabbits exert severe hypercholesterolemia and ACAT inhibition by pactimibe may exacerbate xanthomas. The severity of the xanthomas in the digital joints of the forelegs and hindlegs was evaluated macroscopically by the method of Shiomi et al. (1990), as follows: no lesion (−); slight lesion showing diffuse granular lesions (+); moderate lesion showing one massive lesion with or without diffuse granular lesions (++); and severe lesion showing more than one massive lesion with or without diffuse granular lesions (+++). Xanthomas were evaluated in each leg of each animal, and the incidence was determined by counting the number of legs of each animal with xanthomas for each group. Scores of 0, 1, 2, and 3 were assigned to the classes of −, +, ++, and +++, respectively. The severity index for each group is shown as mean score±S.E.M.

### 2.4. Chemical methods

Blood samples were collected from the ear artery after overnight fasting. Serum total cholesterol and triglyceride levels were measured enzymatically throughout the experiment using an automated analyzer (AU 500, Olympus Optical Company Ltd., Tokyo, Japan). The lipid measurements were performed bimonthly.

The aorta adjacent to that collected for histological evaluation was assayed for the lipid measurements. The adventitial tissue adherent to each region of the aorta was removed. The intimal and medial tissues of these specimens were homogenized in 1 ml water, lyophilized, and then weighed. An aliquot of the homogenate was used for lipid analysis. The aortic cholesterol content was also measured after extraction of lipids using Folch's solution (Folch et al., 1957). Cholesterol determination was carried out using high performance liquid chromatography

Table 2
Lipid composition of aortic lesions in WHHL rabbits

| Treatment group | Cholesterol content (mg/g tissue) | | |
|---|---|---|---|
| | Total | Free | Esterified |
| Aortic arch | | | |
| Control | 211±22 | 87±9 | 124±14 |
| Pactimibe 10 mg/kg | 226±22 | 96±10 | 130±13 |
| Pactimibe 30 mg/kg | 204±25 | 88±10 | 116±15 |
| Thoracic aorta | | | |
| Control | 201±25 | 67±10 | 134±16 |
| Pactimibe 10 mg/kg | 164±15 | 59±6 | 105±10 |
| Pactimibe 30 mg/kg | 142±12 | 52±5 | 90±8[a] |
| Abdominal aorta | | | |
| Control | 203±26 | 79±9 | 123±20 |
| Pactimibe 10 mg/kg | 161±24 | 69±11 | 92±15 |
| Pactimibe 30 mg/kg | 113±27[a] | 48±10 | 64±17[a] |

Data are expressed as mean±S.E.M.
[a] Significantly different from the control group at $P<0.05$ by Dunnett's test.

Table 3
Aortic atherosclerosis in WHHL rabbits

| Treatment group | *n* | Total | Aortic arch | Thoracic aorta | Abdominal aorta |
|---|---|---|---|---|---|
| Lesion coverage, % | | | | | |
| Control | 10 | 68±5 | 91±2 | 78±7 | 47±5 |
| Pactimibe 10 mg/kg | 10 | 64±6 | 85±4 | 74±7 | 42±9 |
| Pactimibe 30 mg/kg | 10 | 65±5 | 83±3 | 79±8 | 37±6 |
| Average intimal thickening, μm | | | | | |
| Control | 10 | 259±24 | 392±35 | 241±28 | 137±18 |
| Pactimibe 10 mg/kg | 10 | 237±22 | 334±41 | 225±30 | 149±34 |
| Pactimibe 30 mg/kg | 10 | 236±25 | 333±29 | 229±30 | 140±20 |

Data are expressed as mean±S.E.M. No significant differences were noted at $P<0.05$.

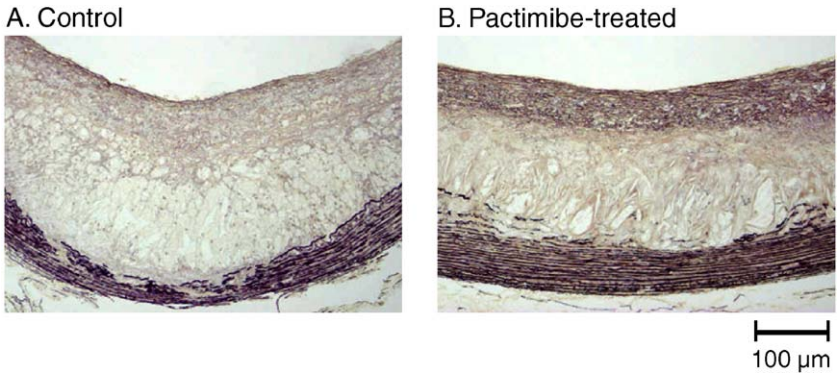



Fig. 2. Photomicrographs showing general morphological appearance of lesions in the proximal thoracic aorta. Panel A: Control. Abundant lipid vacuoles are present throughout the lesion and in portions of the media underlying the lesion. Panels B: Pactimibe when administered at a dose of 30 mg/kg. Note the striking paucity of lipid vacuoles within the lesion, the more collagen fiber and the appearance of the staining compared with that in Panel A. Elastin Van Gieson stain. Bar=100 μm.

(HPLC). HPLC analyses were performed as previously described using a Hitachi Model L-series liquid chromatography system (pump: L-6300; detector: L-4200; integrator: D-2500, Hitachi, Ltd., Tokyo, Japan) (Araki et al., 1990). A reverse phase HPLC column, μBondasphere ($C_{18}$, 3.9×150 mm, 5 μm, 100 Å, Waters Corporation, MA, USA), was used. The column was eluted at a flow rate of 1 ml/min of acetonitrile/2-propanol (1/1, v/v) at 40 °C and monitored at 210 nm. Authentic cholesterol and cholesteryl esters were dissolved in *n*-hexane/2-propanol (3/2, v/v) as external standards (0.1 mg/ml). We defined esterified cholesterol as the sum of cholesterol derived from cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate, and cholesteryl stearate. We also defined total cholesterol as the sum of free and esterified cholesterol. Data are expressed as mg/g dried weight.

### 2.5. Statistical analyses

The results are expressed as the mean±standard error of the mean (S.E.M.). A difference was considered to be statistically significant when the *P* value was less than 0.05. The influence of the treatment with pactimibe on each serum lipid level was assessed using one-way repeated measures analysis of variance (ANOVA) followed by post hoc Dunnett's multiple comparison analyses. The effect of pactimibe on each aortic lipid parameter was assessed using Dunnett's multiple comparison test. All analyses were performed using the Statistical Analysis System (SAS®) Application for Preclinical Study (version 6.12, SAS Institute Inc.).

## 3. Results

### 3.1. Serum lipid concentrations

Table 1 shows the change in serum total cholesterol concentrations throughout the experiment. At the start of the experiment, serum total cholesterol levels (mg/dl) among the groups were equivalent: 944±30, 944±45, and 944±33 for the control, pactimibe 10 mg/kg, and pactimibe 30 mg/kg groups, respectively. After 32 weeks treatment, coordinate age-associated reductions in serum total cholesterol levels were observed as previously reported (Yamada et al., 2004), whereas there were no significant changes among the groups: 611±25, 633±40, and 598±29 at 0, 10, and 30 mg/kg of pactimibe, respectively. The calculated total cholesterol exposure for 32 weeks, measured by the area under the cholesterol concentration–time curve (wk mg/dl), did not show any statistical significance among the groups: 24345±792, 23899±1378, and 22919±747 for the control, pactimibe 10 mg/kg, and pactimibe 30 mg/kg groups, respectively. Moreover, there were no significant changes in phospholipid levels among the groups, whereas significant elevations in triglyceride levels were observed in the pactimibe-treated groups.

### 3.2. Arterial wall cholesterol content

The total, free, and esterified cholesterol contents in the aorta are summarized in Table 2 for all groups. In the aortic arch, there was no apparent effect of pactimibe on the cholesterol

Table 4
Lesion components of atherosclerosis in proximal thoracic aorta of WHHL rabbits

| Treatment group | *n* | Lesion area $\times 10^4$ μm$^2$ | Percent area of lesional components | | | Vulnerbility index |
|---|---|---|---|---|---|---|
| | | | Macrophages [*A*] | Smooth muscle cells [*B*] | Collagen [*C*] | *A*/(*B*+*C*) |
| Control | 10 | 418±64 | 7.0±1.3 | 9.7±0.8 | 16.2±1.0 | 0.27±0.04 |
| Pactimibe 10 mg/kg | 10 | 388±61 | 6.0±1.1 | 12.0±0.9 | 20.5±1.2[a] | 0.19±0.04 |
| Pactimibe 30 mg/kg | 10 | 392±44 | 4.6±1.0 | 12.3±0.5[a] | 31.0±1.3[b] | 0.11±0.02[b] |

Data are expressed as mean±S.E.M.
[a]$P<0.05$, [b]$P<0.01$ vs. respective control by Dunnett's test.

Table 5
Effect of pactimibe on xanthomatosis in WHHL rabbits

| Dose (mg/kg) | No. of rabbits | Incidence (%) | | | Distribution of severity | | | | Severity index |
|---|---|---|---|---|---|---|---|---|---|
| | | Forelegs | Hindlegs | Total | − | + | ++ | +++ | |
| 0 | 10 | 100 (20/20) | 100 (20/20) | 100 (40/40) | 0 | 13 | 11 | 16 | 2.08±0.14 |
| 10 | 10 | 100 (20/20) | 100 (20/20) | 100 (40/40) | 0 | 12 | 16 | 12 | 2.00±0.13 |
| 30 | 10 | 100 (20/20) | 100 (20/20) | 100 (40/40) | 0 | 22 | 9 | 9 | 1.68[a]±0.13 |

Severity of xanthoma: (−) no lesion; (+) slight lesion showing diffuse granular lesions; (++) moderate lesion showing one massive lesion with or without diffuse granular lesions; (+++) severe lesion showing more than one massive lesion with or without diffuse granular lesions.
Data in parentheses represent the number of injured legs/number of examined legs.
Severity index in each group is shown as a mean±S.E.M.
[a] $P<0.05$ compared with the control group by non-parametric Williams' test.

contents. In the thoracic aorta, pactimibe at 30 mg/kg significantly reduced the esterified cholesterol content by 33% relative to the control group. In addition, a 22% reduction in free cholesterol content and a 29% reduction in total cholesterol content were noted in the pactimibe 30 mg/kg-treated group, although these decreases were not statistically significant. In the abdominal aorta, pactimibe at 30 mg/kg significantly reduced the total and esterified cholesterol contents by 44% and 48%, respectively, compared with the respective control group. Free cholesterol contents were reduced by 39%, although without statistical significance in the pactimibe 30 mg/kg-treated group.

### 3.3. Morphometric analyses

The percentage area of the aorta covered by atherosclerotic lesions and the average intimal thickening of the aorta were assessed and are shown in Table 3. There were no significant differences among the groups either in the percentage lesion area in any portions or in the average intimal thickening. Fig. 2 shows photomicrographs of typical aortic atherosclerosis in the proximal thoracic aorta. The sizes of the lesions in the groups treated with pactimibe were similar to those in the control group. Further histopathological examination of the proximal thoracic lesions revealed that pactimibe administration altered plaque composition in the proximal thoracic aorta displayed as area of monocyte-macrophage foam cells, smooth muscle cells, and collagen fibers (Table 4). Pactimibe reduced the percent macrophage area from 7.0±1.3% (control) to 6.0±1.1% (10 mg/kg) and 4.6±1.0% (30 mg/kg), although it did not achieve statistical significance. Pactimibe caused a dose-dependent increase in the ratio of the smooth muscle cells (12.0±0.9% and 12.3±0.5%, $P<0.05$, at 10 and 30 mg/kg, respectively versus 9.7±0.8% in the control) as well as the area of collagen to the lesion (20.5±1.2%, $P<0.05$, and 31.0±1.3%, $P<0.05$, at 10 and 30 mg/kg, respectively versus 16.2±1.0% in the control). The vulnerability index was reduced in a dose-dependent manner by 30% and 59% ($P<0.05$) at 10 and 30 mg/kg of pactimibe, respectively, from the control. Table 5 shows the development of xanthomas in WHHL rabbits. The incidence of xanthomas was 100% in all the groups. The severity indices of xanthomatosis were significantly lower in the pactimibe-treated groups: 2.08±0.14, 2.00±0.13, and 1.68±0.13 ($P<0.05$) for the control, pactimibe 10 mg/kg, and pactimibe sulphate 30 mg/kg groups, respectively.

## 4. Discussion

Our results demonstrate that although pactimibe did not impact atherosclerotic lesion coverage or intimal thickening in the aorta, it reduced esterified cholesterol in plaque without change in serum total cholesterol level in WHHL rabbits. Moreover, plaque components were altered to a potentially more stable plaque phenotype, suggesting that novel ACAT inhibitor pactimibe directly stabilizes atherosclerotic lesions by limiting foam cell formation and reducing esterified cholesterol content in plaque in WHHL rabbits.

The crux of anti-atherosclerosis activity in WHHL rabbits is serum total cholesterol reduction, as evidenced by several cholesterol-lowering agents, such as HMG-CoA reductase inhibitor pravastatin (Harsch et al., 1997; Shiomi et al., 1999, 1990; Watanabe et al., 1988) and cholestyramine (Subbiah et al., 1987). In contrast to other ACAT inhibitor studies (Kogushi et al., 1996; Matsuo et al., 1995), pactimibe in our study did not affect serum total cholesterol levels or cause significant reduction in plaque size and/or lesion coverage in WHHL rabbits, suggesting weaker ACAT inhibitory activity. E-5324 reduced both free and esterified cholesterol contents in the aorta in WHHL rabbits, corroborating our own observations, indicating that pharmacological inhibition of macrophage ACAT could prevent cholesteryl ester formation in atherosclerotic lesions although not free cholesterol increase in plaque.

The key observation of this study is that pactimibe stabilized plaque independent of cholesterol lowering in the aorta via drastic reduction in the esterified cholesterol content, the end product of the ACAT reaction.

Paradoxically, complete ACAT1 deficiency in macrophages promoted rather than inhibited atherosclerotic lesion formation without affecting plasma cholesterol levels in studies using bone marrow transplantation in both hypercholesterolemic LDL receptor knockout mice (Fazio et al., 2001) and apo E knockout mice (Su et al., 2005), suggesting that selective, potent ACAT1 inhibition in macrophages would not be a beneficial strategy for treating or preventing atherosclerosis in the setting of severe hypercholesterolemia. Warner et al. (1995) reported that treatment of mouse peritoneal macrophages with ACAT inhibitor Sandoz 58-035 or Pfizer CP-113818 with acetylated LDL loading without an appropriate cholesterol acceptor in the culture media increased intracellular free cholesterol levels and

promoted apoptosis. Kellner-Weibel et al. (1998) also demonstrated free cholesterol accumulation in macrophages with Sandoz 58-035 treatment. Thus, potent ACAT inhibition can adversely affect atherosclerotic progression via severe membrane perturbation, possibly due to increased accumulation of cytotoxic free cholesterol, as reported by Tabas (2002, 2005), contributing to the deposition of free cholesterol within the necrotic core of more advanced fibrous plaque.

However, pactimibe did not increase free cholesterol content in this study, possibly due to partial pharmacological inhibition. Theoretically, ACAT inhibition may release free cholesterol in the cells, consequently promoting apolipoprotein-mediated cholesterol efflux and reverse cholesterol transport to the liver. Release of cellular cholesterol is well documented by Yokoyama (1998, 2000). Moreover, several studies have shown that treatment with ACAT inhibitors enhances cholesterol efflux in macrophages (Sugimoto et al., 2004) and fibroblasts (Yamauchi et al., 2004), which, in turn, attenuates the toxic effects of overloaded cholesterol on macrophage function, limits foam cell formation, and improves cholesterol removal from the vessel wall. These findings support the hypothesis that pharmacological inhibition of arterial ACAT could prevent lipid core formation and promote plaque destabilization, although the precise underlying mechanism(s) of plaque stabilization remains unclear.

Complete deletion of ACAT was reported to cause xanthomatosis from overwhelming free cholesterol deposition in the skin and brain (Accad et al., 2000; Yagyu et al., 2000). Thus, we investigated xanthomatosis after pactimibe treatment in WHHL rabbits which exert severe hypercholesteremia. Of note, pactimibe at 30 mg/kg significantly reduced xanthomatosis in comparison with the control group, suggesting that partial inhibition of macrophage ACAT may not exacerbate xanthomatosis even under hypercholesterolemic conditions.

Recently, in a clinical phase IIb/III intravascular ultrasound (IVUS) study with pactimibe, the ACTIVATE study, pactimibe failed to reduce atherosclerotic disease progression over 18 months when compared with usual care for coronary artery disease patients (Nissen et al., 2006). Nissen et al. (2006) suggested that increased levels of free cholesterol within the macrophage might cause apoptosis, creating necrosis within the lesion in humans. IVUS results with Pfizer's ACAT inhibitor, avasimibe, in the A-PLUS trial (Tardif et al., 2004) support this hypothesis. However, IVUS can only delineate size of plaque, not content. Based on the result of our study in WHHL rabbits, pactimibe may act toward stabilizing plaque without accumulation of free cholesterol, providing new implications for the potential for plaque stabilization treatment via macrophage ACAT inhibition in humans.

An unexpected observation in our study was that pactimibe oral administration to WHHL rabbits fed a basal diet elevated serum triglyceride levels in contrast to other systemically bioavailable ACAT inhibitors (Kogushi et al., 1996; Matsuo et al., 1995). This discrepancy is likely due to decreased VLDL clearance rather than enhanced VLDL triglyceride secretion, which was not found in our preliminary experiment in WHHL rabbits (Triton WR-1339 method; data not shown), for the following reasons. Triglyceride elevation accompanying reduced VLDL triglyceride clearance has been found in ACAT2-apolipoprotein (apo) E double knockout mice, primarily due to replacement of cholesterol esters with triglycerides in the apo B-containing lipoprotein core (Willner et al., 2003). Moreover, the concentration and composition of the VLDL lipid core may modify apolipoprotein orientation, hence altering receptor affinity (Sehayek and Eisenberg, 1994). Reduction in the cholesteryl ester content in VLDL particles quite likely decreases the rate of hepatic uptake, which may also be affected by the lipid composition of the particles.

Elevated plasma triglyceride levels are considered a potential risk factor for atherosclerosis. However, study findings in ACAT2-Apo E double knockout mice strongly suggest that triglyceride-rich apo B-containing lipoproteins are not as atherogenic as those that are rich in cholesterol esters. We could not rule out the effect of triglyceride elevation on lesion formation but it would be negligible in view of the relatively low change in serum triglyceride levels (around 1.5 times elevation from the baseline, see Table 1).

In conclusion, we demonstrated that long-term pactimibe treatment stabilized atherosclerotic plaque in WHHL rabbits. The mechanisms of the plaque stabilizing effect of pactimibe in WHHL rabbits presumably involve several properties of its pharmacological profiles, including its ability to 1) inhibit foam cell formation, 2) promote cellular cholesterol efflux and reverse cholesterol transport, and/or 3) modulate the function of macrophages, smooth muscle cells, and lymphocytes. These findings reveal that intervention aimed at improving the vulnerability of plaques to disruption, i.e. the concept of plaque stabilization, may reduce the risk of acute coronary syndromes even without lipid lowering.

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