Regression of Atherosclerosis in Apolipoprotein E-Deficient Mice is Feasible Using High-Dose Angiotensin Receptor Blocker, Candesartan

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Aim: Clinical studies have suggested that renin-angiotensin inhibitors are effective for the prevention of atherosclerosis progression, but the results for the regression of established lesions are equivocal. The aim of this study was to examine the effects of different doses of the angiotensin receptor blocker (ARB) candesartan on the regression of atherosclerosis and lipid-induced nephropathy in apolipoprotein E (apoE)-deficient spontaneously hyperlipidemic (SHL) mice.

Methods and Results: Male SHL were given an atherogenic diet together with salt loading to induce atherosclerosis. The mice were then treated with various doses of candesartan (0-50 mg/kg/d) for 12 weeks. Treatment with high-dose candesartan caused clear regression of atherosclerotic plaques in the aorta, which was not observed with normal-dose candesartan. Biglycan and ACAT1 expression were significantly decreased, and aortic free cholesterol: cholesterol ester ratios were increased in these mice. Treatment of cultured THP-1 macrophages in vitro with candesartan resulted in a similar decrease in ACAT1 expression. In the kidney, glomerular lipid accumulation, mesangial expansion, and albuminuria were significantly regressed after treatment with high-dose candesartan, while biglycan and ACAT1 expressions were decreased.

Conclusion: These results suggest that regression of established atherosclerosis lesions in ApoE-deficient mice is feasible using high-dose candesartan, by mechanisms involving (i) a decrease in the lipid-retaining proteoglycan biglycan, and (ii) suppression of ACAT1 expression resulting in increased free cholesterol for lipid release.


Key words: Angiotensin receptor blocker, Candesartan, Regression, Biglycan, ACAT1

Introduction

Atherosclerosis is a leading cause of morbidity and mortality throughout the world1). Regression, or reversal of atherosclerotic lesions, could result in a marked decrease in coronary heart disease, stroke, and peripheral vascular disease2, 3); therefore, the development of methods for atherosclerosis regression represents an important therapeutic goal in the management of cardiovascular diseases.

Previous studies have suggested that the renin-angiotensin system (RAS) plays an important role in the onset and progression of atherosclerosis. Experimental studies have shown that treatment with RAS inhibitors prevents the formation of atherosclerotic lesions in animal models4, 5). Moreover, the use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) has been shown in clinical studies to inhibit the progression of carotid medial thickening, decrease oxidative stress and inflammation, and improve endothelial function6).

Interestingly, several preliminary studies have suggested that use of ARBs may cause a decrease or regression of plaque volumes. In an intravascular ultrasound study, Waseda et al. reported that treatment of patients with ARB was associated with a significant decrease in plaque volume7). Similar results were reported in the Multicenter Olmesartan atherosclero-
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Materials and Methods

Animal Treatment Protocols

The studies were conducted using 4-week-old male spontaneously hyperlipidemic (SHL) mice (C57BL/6.KOR/StmSc-Apoeshl), which were obtained commercially from Sankyo Laboratory Service Corporation (Tokyo, Japan). All experiments were performed in accordance with the Animal Experimentation Guidelines of Keio University School of Medicine. Candesartan cilexetil (TCV-116) was a kind gift from Takeda Pharmaceutical Inc. (Tokyo, Japan).

SHL mice were divided into 6 groups as follows (n = 8 per group): Group 1 was fed a normal rodent diet. Groups 2-6 were fed an atherogenic diet (15.0% lard and 0.15% cholesterol) obtained from Crea Japan (Tokyo, Japan), together with 1% NaCl in the drinking water for 12 weeks (from 5 to 16 weeks old). Group 2 was sacrificed at 16 weeks old before starting the ARB candesartan cilexetil treatment. Group 3, 4, 5 and 6 were treated for 12 weeks (from 16 to 28 weeks old) with ARB candesartan cilexetil was dissolved in the drinking water to deliver a dose of 0, 1, 5, 10, 50 mg/kg/day, respectively, as described by us previously. All groups were returned to a normal diet with normal drinking water at age 16 weeks. Group 1 was sacrificed at 28 weeks old at the same time as Groups 3-6.

Biochemical Studies and Blood Pressure Measurement

Blood was collected by heart puncture after an overnight fast, centrifuged at 850 g for 15 min at 4 °C, and stored at −20 °C until assay. Serum total cholesterol and HDL cholesterol were measured enzymatically. Urine collections were performed in metabolic cages, and urine albumin concentrations were determined by a direct competitive ELISA (Albuwell, Exocell, PA). Systolic blood pressures were estimated by indirect tail-cuff plethysmography using a Softron BP-98A manometer (Softron Inc., Tokyo, Japan). The protocol for blood pressure measurement was as follows: Preliminary blood pressure measurements were performed on two separate occasions to train the mice and to confirm that individual blood pressure variability was low. For the measurement of final blood pressures, the mean of 3-5 independent measurements was recorded at age 28 weeks, as recommended by the manufacturer.

Histological Analysis of Aortas and Kidneys

The heart and the aorta were immediately removed en bloc with a wide range of surrounding tissues. The aorta (from the ascending aorta to the abdominal aorta) was fixed in 4% paraformaldehyde, after which the whole aorta was opened longitudinally for en face Sudan IV staining. Sudan IV-positive areas in the aorta were quantified using Adobe Photoshop data analysis software (Adobe Systems, San Jose, CA, USA). For the assessment of atherosclerosis in the aortic root, heart sections containing the aortic root were fresh-frozen in OCT compound, and then sectioned using a cryotome prior to oil red O staining. For the assessment of renal injury, the kidneys were removed and fixed in 4% paraformaldehyde, and then embedded in paraffin blocks for periodic acid-Schiff (PAS) staining. Other kidney samples for oil red staining and immunofluorescence analysis were fresh-frozen in OCT compound.

Immunofluorescence Staining and Western Blotting

Immunofluorescence staining and quantification of biglycan, ACAT1, and ABCA1 expression were performed on the cryostat sections using polyclonal anti-biglycan antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-ACAT1 antibodies
The human monocytic leukemia cell line, THP-1 (obtained from DS Pharma Biomedical, Osaka, Japan) was grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. To induce macrophage phenotype differentiation, phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) was added to the culture medium to induce differentiation into macrophages. In some experiments, THP-1 cells were stimulated with 10 μg/mL oxidized LDL (ox-LDL; Cell Biolabs Inc., San Diego, CA, USA). The differentiated macrophages were treated with candesartan (CV-11974, active metabolite of candesartan cilexetil, 10^{-6} mol/L) for 24 hours, and then ACAT1 and ABCA1 expressions were analyzed by Western blot analysis.

**Lipid Extraction and Analysis**

Total lipid was extracted from fresh-frozen samples of thoracic aortas or the renal cortex by the method of Bligh and Dyer. Total cholesterol and free cholesterol contents were measured using the cholesterol E test kit and free cholesterol E test kit (Wako Chemicals, Tokyo, Japan).

**Cell Culture**

The human monocytic leukemia cell line, THP-1 (obtained from DS Pharma Biomedical, Osaka, Japan) was grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. To induce macrophage phenotype differentiation, phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) was added to the culture medium to induce differentiation into macrophages. In some experiments, THP-1 cells were stimulated with 10 μg/mL oxidized LDL (ox-LDL; Cell Biolabs Inc., San Diego, CA, USA). The differentiated macrophages were treated with candesartan (CV-11974, active metabolite of candesartan cilexetil, 10^{-6} mol/L) for 24 hours, and then ACAT1 and ABCA1 expressions were analyzed by Western blot analysis.

**Statistics**

Results are expressed as the mean ± SEM. Statistical comparisons were made by ANOVA followed by Schef-fe’s post-hoc test. $P < 0.05$ was considered significant.
mal dose (1 mg/kg/day in rodents16)) for 12 weeks did not cause a significant change in the Sudan IV-stained area in the aorta. In contrast, treatment with high doses of candesartan (10 and 50 mg/kg/d) caused a significant decrease in aortic atherosclerotic lesions. Similar results were found for oil red O-stained areas in the aortic roots. Aortic cholesterol contents were also significantly decreased in high-dose ARB-treated mice. In contrast, no significant changes in systolic blood pressure, serum total cholesterol, or HDL-cholesterol were found in the different groups (Table 1). Furthermore, the free cholesterol: cholesterol ester ratios were significantly increased in the high-dose candesartan-treated group, but not in the normal-dose candesartan-treated group (Fig. 1C).

Effects of Different doses of ARB on Lipid-Related Factors in the Aorta of SHL Mice

To examine the possible role of the lipid-retaining proteoglycan biglycan in the regression of atherosclerosis, the expression of biglycan in the aortic plaques was examined by immunofluorescence staining. It was found that the expression of biglycan was significantly reduced by high-dose candesartan treatment, but not by low-dose candesartan, and a similar relation was seen with ApoB staining (Fig. 2A). Because the free cholesterol: cholesterol ester ratios were increased in candesartan-treated groups, we also examined ACAT1 expression in the different groups. As shown in Fig. 2B, the expression of ACAT1 was significantly decreased in the aortic plaques of candesartan-treated mice. In contrast, no significant differences were found in the expression of ATP-binding cassette transporter A1 (ABCA1) or for ABCG1 (data not shown). Double immunostaining revealed that biglycan expression co-localized with ApoB staining, and ACAT1 and ABCA1 were co-expressed with CD68-positive macrophages in the aortic plaques (Fig. 2C). The changes in ACAT1 and ABCA1 expression were confirmed by Western blotting (Fig. 2D). To confirm these actions of candesartan in vitro, we used the cultured human macrophage cell line THP-1, which was differentiated using the phorbol ester PMA. It was found that treatment with candesartan (10−6 M) induced a decreased expression of ACAT1 but not ABCA1. Similar results were found in THP-1 cells stimulated with ox-LDL (Fig. 2E, F).

Results

Differential Effects of High-Dose Versus Normal-Dose ARB on Aortic Atherosclerotic Lesions of SHL Mice

SHL mice were fed an atherogenic diet with salt loading from 5 to 16 weeks of age. Mice in Group 2 were sacrificed at age 16 weeks, and lipid accumulation in the aorta and the aortic root were assessed by en face staining with Sudan IV and oil red O staining, respectively, to confirm that atherosclerosis had already developed at this age (Fig. 1A, B). Next, the effects of different doses of the ARB candesartan on atherosclerotic lesions were examined by treating the mice with candesartan from age 16 to age 28 weeks. It was found that treatment with candesartan at a normal dose (1 mg/kg/day in rodents16) for 12 weeks did not cause a significant change in the Sudan IV-stained area in the aorta. In contrast, treatment with high doses of candesartan (10 and 50 mg/kg/d) caused a significant decrease in aortic atherosclerotic lesions. Similar results were found for oil red O-stained areas in the aortic roots. Aortic cholesterol contents were also significantly decreased in high-dose ARB-treated mice. In contrast, no significant changes in systolic blood pressure, serum total cholesterol, or HDL-cholesterol were found in the different groups (Table 1). Furthermore, the free cholesterol: cholesterol ester ratios were significantly increased in the high-dose candesartan-treated group, but not in the normal-dose candesartan-treated group (Fig. 1C).

Effects of Different doses of ARB on Glomerular Lesions in SHL Mice

Treatment of SHL mice with an atherogenic diet with salt loading from 5 to 16 weeks of age caused a significant increase in kidney glomerular lipid accumulation and mesangial expansion (Fig. 3A-C). Similar to the aorta, treatment with candesartan for 12 weeks caused a reduction in the number of oil red O-positive glomeruli, which was not statistically significant from the normal dose of candesartan (1 mg/kg/d), but was clearly seen with higher doses of candesartan (10 and 50 mg/kg/d). Quantitative assessment of the PAS-positive stained area revealed that the decrease in glomerular lipid accumulation was associated with a reduction in the mesangial matrix score in mice treated with higher doses of candesartan (10 and

Table 1. Effects of ARB on body weight, systolic blood pressure, serum total cholesterol, and HDL-cholesterol in SHL mice

<table>
<thead>
<tr>
<th>Atherogenic diet</th>
<th>−</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
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</thead>
<tbody>
<tr>
<td>Candesartan (mg/kg/day)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>28</td>
<td>16</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>34.8±1.5</td>
<td>34.4±1.7</td>
<td>36.2±1.7</td>
<td>37.2±1.4</td>
<td>35.8±1.5</td>
<td>35.0±1.4</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>107.4±5.6</td>
<td>103.6±4.7</td>
<td>114±5.7</td>
<td>108.4±7.6</td>
<td>114.8±8.0</td>
<td>112.6±5.7</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>528±96</td>
<td>540±198</td>
<td>613±62</td>
<td>497±17</td>
<td>620±113</td>
<td>588±62</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>15.0±4.5</td>
<td>16.4±2.4</td>
<td>13.0±2.0</td>
<td>17.0±1.2</td>
<td>13.8±2.4</td>
<td>13.0±3.0</td>
</tr>
<tr>
<td>Aortic cholesterol content (ng/mg)</td>
<td>273±46*</td>
<td>459±88</td>
<td>601±119</td>
<td>445±71</td>
<td>392±41</td>
<td>335±64*</td>
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*p<0.05 vs atherogenic diet (+) Candesartan 0, 28 weeks old
ABCA1 in the glomeruli were examined. As shown in Fig. 4A, B, the glomerular expression of biglycan and ACAT1 was significantly decreased in the high-dose candesartan-treated group, whereas the expression of ABCA1 was not significantly different between the groups, as in the aortic plaques. In the case of the renal glomeruli, both ACAT1 and ABCA1 expressions were found to be localized predominantly in the mesangial area, rather than CD68-positive macrophages (Fig. 4C).

**Discussion**

Atherosclerosis, which occurs as a result of excessive lipid accumulation in aortic plaques, is usually...
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considered a chronic and progressive process. Historically, the idea that atheromatous lesions may decrease in size has met with considerable resistance and skepticism\(^24, 25\); therefore, most previous pharmacological interventions have focused on the ‘prevention’ of atherosclerosis, that is, inhibition of the progression of atherosclerotic lesions. There have been fewer studies focusing on the mechanisms of ‘regression’, or the reversal of established atherosclerosis in animal models.

In one of the first studies on atherosclerosis regression, Friedman et al. treated cholesterol-fed rabbits with bolus injections of phosphatidylcholine and found a significant reduction in atheromatous plaques\(^26\). Recently, several clinical trials have examined the possible regression of atherosclerotic lesions in humans. Nissen et al. investigated the effects of recombinant ApoA-I Milano given intravenously for 5 weeks, and found a significant decrease in atheroma volume\(^27\). In the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) study, the effect of high-dose statin therapy was compared with a conventional, less potent statin regimen. Patients treated with the conventional regimen exhibited a statistically significant progression of atheroma volume, whereas the high-dose statin group did not\(^28\). In the A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden (ASTEROID) trial, all patients received high-dose statin therapy for 24 months, which resulted in a significant regression of coronary atherosclerosis\(^29\). The results of these studies suggest that the development of effective methods to cause the regression of atherosclerosis may be considered an achievable goal.
arterial wall, and ii) efflux of cholesterol from plaques. Biglycan is a small leucine-rich proteoglycan which is expressed in the arterial wall and is thought to play a central role in the retention of atherogenic lipoproteins in atherosclerotic lesions. We and others have shown that angiotensin II causes an increase in biglycan synthesis, while treatment with ARB inhibits biglycan expression, which may contribute to the anti-atherogenic effect of RAS inhibition. These reports suggest the possibility that the regression of atherosclerosis induced by high-dose candesartan treatment may be mediated by decreased biglycan expression, resulting in decreased accumulation of lipids in the arterial wall. In this study, we found that high-dose candesartan treatment caused a reduction of biglycan accumulation in both the aorta and the kidney glomeruli. The expression of biglycan was co-localized with ApoB expression in the aorta. In addition, a parallel relation was found between the expres-

Fig. 4. Changes of lipid-related factors in the kidneys of SHL mice with different doses of candesartan. (A) Quantification of biglycan-, ACAT1- and ABCA1-stained areas in the glomeruli of SHL mice. (B) Representative photomicrographs of biglycan, ACAT1 and ABCA1 immunofluorescence staining. (C) Representative immunofluorescence staining of CD68 (green) and ACAT1 (red) or ABCA1 (red) in the kidneys of SHL mice with an atherogenic diet together with salt loading. Abbreviations as in Fig. 1.

in order to attenuate the growth in the increasing number of patients with cardiovascular diseases.

In this study, we compared the effect of different doses of the ARB candesartan on the regression of atherosclerosis in SHL mice. These SHL mice are known to have apoE gene mutations with more severe hypercholesterolemia and xanthoma, and generally milder atherosclerosis than apoE-null mice. Preliminary experiments revealed that combining an atherogenic diet with salt loading in the drinking water resulted in the formation of severe atherosclerotic plaques in this model. Our results suggested that treatment for 12 weeks with high-dose candesartan resulted in the significant regression not only of aortic atherosclerotic lesions, but also of glomerular lipid deposits in the kidney.

It has been suggested that several mechanisms may contribute to atherosclerosis regression, including i) decreased retention of apoB lipoproteins within the arterial wall, and ii) efflux of cholesterol from plaques. Biglycan is a small leucine-rich proteoglycan which is expressed in the arterial wall and is thought to play a central role in the retention of atherogenic lipoproteins in atherosclerotic lesions. We and others have shown that angiotensin II causes an increase in biglycan synthesis, while treatment with ARB inhibits biglycan expression, which may contribute to the anti-atherogenic effect of RAS inhibition. These reports suggest the possibility that the regression of atherosclerosis induced by high-dose candesartan treatment may be mediated by decreased biglycan expression, resulting in decreased accumulation of lipids in the arterial wall. In this study, we found that high-dose candesartan treatment caused a reduction of biglycan accumulation in both the aorta and the kidney glomeruli. The expression of biglycan was co-localized with ApoB expression in the aorta. In addition, a parallel relation was found between the expres-
Atherosclerosis Regression and Candesartan was significantly lower than in control and standard-dose candesartan-treated groups, and the ratios of free cholesterol contents to cholesterol ester in high-dose candesartan-treated aortas were significantly higher. Moreover, treatment of cultured macrophages with candesartan resulted in a decrease in ACAT1 expression. Macrophages have been shown to induce the full expression of the components of the RAS, including the AT1 receptor. These results are consistent with the possibility that candesartan treatment inhibits the action of locally generated angiotensin II, and therefore suppresses ACAT1 expression in macrophages.

Concerning the mechanisms of the effects of high-dose candesartan, it has been shown that maximal inhibition of the AT1 receptor is achieved at high concentrations of candesartan (1 μM or greater), which is higher than the plasma concentration achieved with normal-dose candesartan; therefore, the effects of high-dose candesartan could be explained by more complete inhibition of the AT1 receptor than with normal-dose candesartan. Interestingly, it has been shown that candesartan has multiple pleiotropic actions, some of which could be mediated by AT1 receptor-independent mechanisms. The possibility that such mechanisms could contribute to the effects seen in this study cannot be completely ruled out.

Concerning the glomerular lipid deposits in hyperlipidemic mice, Wen et al. reported that glomerular lesions in ApoE-deficient mice were characterized...

![Diagram of lipid retention and release](Fig. 5A)

**Fig. 5.** Possible mechanisms of regression of atherosclerosis by high-dose candesartan treatment, involving (A) decreased lipid retention (B) increased lipid release.

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**Diagram Description:**

- **A:** **Lipid Retention**
  - Side chain
  - ApoB
  - Lipid
  - Biglycan (Decreased)
  - Candesartan

- **B:** **Lipid Release**
  - Free cholesterol
  - ABC transporter
  - Lipid
  - ACAT1 (Decreased)
  - Cholesterol ester (Decreased)

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Concerning the glomerular lipid deposits in hyperlipidemic mice, Wen et al. reported that glomerular lesions in ApoE-deficient mice were characterized...
not only by lipid deposits, but also by increased deposition of glomerular matrix \[46\]. Similarly, Tomiyama-Hanayama et al. reported increased mesangial proliferation and matrix expansion in ApoE-deficient mice \[47\]. It has been reported that hyperlipidemia accelerates glomerulosclerosis, whereas manipulations to prevent intracellular lipid accumulation improve the progression of renal injury \[48\]. In our study, we found that lipid deposition, biglycan expression, and ACAT1/ABCA1 expression were predominantly co-localized to the mesangial area, and that candesartan treatment resulted in a decrease in lipid deposition. Therefore, high-dose candesartan treatment may have caused a decrease in mesangial lipid deposition by similar mechanisms as in the aortic macrophages, resulting in decreased mesangial expansion, and a significant reduction in albuminuria.

Concerning the dose of candesartan used in this study, candesartan has been shown to have an antihypertensive effect at 1 mg/kg/day in hypertensive rats \[35\] and hypertensive mice \[49\]. The dose of 50 mg/kg/day was 50 times the rodent antihypertensive dose; therefore, this was defined as ‘high-dose’ candesartan, as in our previous study \[16\]. In humans, the maximal antihypertensive dose in Japan is 12 mg/day. Interestingly, several studies have been performed to examine the effects of ‘ultrahigh’ doses of ARB (candesartan 16-128 mg/day or irbesartan 300-900 mg/day) in humans. The use of these high doses of ARB was associated with decreased proteinuria without dosage-related side effects \[50, 51\]; therefore, clinical use of high doses may be feasible. These studies also showed that the antiproteinuric effect of ARBs may be independent of the antihypertensive effect. In this study, the ARB candesartan had an antithrombotic effect without a significant change in blood pressure, which is consistent with the notion that some actions of ARB may be mediated by blood pressure-independent mechanisms.

As mentioned in the introduction, several clinical studies have already suggested that use of ARBs may cause a decrease or regression of atherosclerosis \[7, 8\], but this has not been confirmed in other studies \[12, 13\]. To our knowledge, all previous clinical studies have been performed with a normal dose of ARB. In this study, we found that robust regression of atherosclerosis was only found in mice treated with high-dose ARB candesartan, suggesting that different doses may lead to different results.

In conclusion, the results of this study suggest that high-dose candesartan treatment causes regression of established atherosclerosis lesions in SHL mice, by mechanisms which may involve (i) decreased expression of the lipid-retaining proteoglycan biglycan, and (ii) reduced expression of the cholesterol converting enzyme ACAT1, resulting in a relative increase in free cholesterol for lipid release. These results may be important for designing therapies for the ultimate goal: to induce the regression of atherosclerotic disease and to substantially reduce stroke and coronary heart disease throughout the world.

Acknowledgements

The authors are grateful to Dr. Masataka Sata (Department of Cardiovascular Medicine, Institute of Health Biosciences, University of Tokushima Graduate School, Japan) and his collaborators at Tokyo University (Ms Yumi Kato and Ms Yumi Sugawara) for assistance with the quantitation of atherosclerotic plaques, to Dr. Shinji Kume (Department of Medicine, Shiga University of Medical Science, Japan) for assistance with kidney lipid quantitation, and Dr. Mitsuhiro Watanabe (Keio University) for helpful discussion about ACAT1 and ABCA1 expression.

Sources of Funding

This study was supported by a Grant-in-Aid for JSPS Fellows (2155542) and Grants for Scientific Research (20590984, 2155542, 20680105) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, the Nateglinide Toyoshima Research and Education Fund, and the Salt Science Foundation, Tokyo, Japan.

Disclosures

None.

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