A Matrix Metalloproteinase Inhibitor, ONO-4817, Suppresses the Development of Aortic Intimal Hyperplasia in Experimental Hyperlipidemic Rabbit

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SUMMARY

Inhibition of matrix metalloproteinases (MMPs) would be expected to suppress atherosclerotic neointimal proliferation and thus limit atheromatous plaque progression, but this has not yet been demonstrated morphologically in atherosclerotic intimal hyperplasia induced by cholesterol loading in experimental animals. We therefore investigated whether a broad-spectrum MMP inhibitor (MMPi), ONO-4817, could inhibit the development of intimal hyperplasia in male hyperlipidemic rabbits ($n = 6$) fed laboratory chow supplemented with 1% cholesterol for 2 months followed by a 1% cholesterol diet plus 100 mg/kg ONO-4817 for another month (Chol + ONO group). Control animals ($n = 6$) received no ONO-4817. When the aortas were studied both histologically and immunohistochemically, intimal hyperplasia was inhibited in Chol + ONO rabbits. The distribution of macrophages and MMP-12 in the hyperplastic tissue of the Chol + ONO rabbits was limited to the luminal side of the lesions. No such limitation in the distribution of macrophages and MMP-12 was observed in the control group. The distribution of smooth muscle cells in the hyperplastic tissue was not different between the Chol + ONO and control groups. However, the distribution of MMP-2 and MMP-12 was limited to the luminal side of lesions in the Chol + ONO group. This is the first reported evidence that an MMPi can suppress the development of intimal hyperplasia in hyperlipidemic rabbits. (Int Heart J 2007; 48: 369-378)

Key words: Atherosclerosis, Extracellular matrix, Matrix metalloproteinase, ONO-4817

MATRIX metalloproteinases (MMPs) have been shown to be expressed in vulnerable regions of atherosclerotic plaques[1-3] and are considered crucial in the progression and destabilization of atherosclerotic plaques.[4,5] Therefore, inhibiting the activity of these enzymes should limit the enlargement of these lesions and

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enhance their stability.[4-6] Several studies with synthetic matrix metalloproteinase inhibitors (MMPi) have been conducted to test this hypothesis, but no convincing morphologic data have yet been obtained.[7-9]

ONO-4817, a newly synthesized MMPi, is active as an MMPi after oral administration, and has a partially selective inhibitory spectrum (MMP-2, MMP-8, MMP-9, MMP-12, and MMP-13 but not MMP-1 or MMP-7).[10] ONO-4817 is reported to inhibit metastasis in a variety of tumors[11-15] and also is reported to be able to inhibit neointimal formation by intimal injury in hypercholesterolemic hamster[16] and allograft vasculopathy.[17] The amount of MMP-2, MMP-9, and MMP-12 in neointimal lesions formed in hypercholesterolemic hamsters is reported to be decreased by ONO-4817.[16]

In this study, we present morphologic evidence that a broad-spectrum MMP inhibitor, ONO-4817, can suppress the development of atherosclerotic intimal hyperplasia induced by dietary hypercholesterolemia in experimental rabbits.

METHODS

Animal preparation: Twelve 3-week-old Japanese white male rabbits were used. The rabbits were fed laboratory chow supplemented with 1% cholesterol to induce atherosclerotic intimal hyperplasia in the aorta. The Institutional Animal Care and Use Committee of the National Defense Medical College approved all of the animal protocols used in this study.

Lesion induction and treatment with ONO-4817: The twelve rabbits were fed laboratory chow supplemented with 1% cholesterol for 2 months. Among these rabbits, 6 were fed the same laboratory chow without any other interventions throughout the experimental period (control group). After 2 months of this 1% cholesterol diet, 6 other rabbits received 100 mg/kg of ONO-4817 orally in distilled water containing 0.5% sodium carboxymethyl cellulose for 1 month daily, while the 1% cholesterol feedings were continued (Chol + ONO group). After beginning ONO-4817 administration, body weight was recorded once a week to adjust the dose of ONO-4817 and to compare the body weights between the control and Chol + ONO groups. Statistical comparisons of the body weight between 2 groups were performed using Welch's *t*-test.

Serum lipid profiles of animals: Three representative rabbits in both groups were selected to monitor the serum lipid profiles (total cholesterol, HDL-cholesterol, triglycerides) during ONO-4817 administration. Statistical comparisons of each profile between the 2 groups were performed using Welch's *t*-test.

Histologic and immunohistochemical analyses: After 3 months of the treatment outlined above, the aortas were removed from the rabbits, fixed with 4% paraformaldehyde in phosphate-buffered saline at pH 7.2, and embedded in par-

affin. Serial 4-μm sections obtained from these tissues specimens were used for the histologic and immunohistochemical analyses. Routine hematoxylin and eosin (H and E) staining of transversely sectioned aortas was performed. After acquiring digital images of routinely H and E stained tissue by microscopy, the ratio of neointimal lesion to total cross sectional area of the aorta was analyzed using a computer image graphic analysis software program (VM32; Rise Corporation, Sendai, Japan). Five representative lesions located after the branching of the renal arteries were analyzed from 3 aortas (30 lesions total) and the results are expressed as the mean ± SD. Statistical comparisons between the 2 groups were performed using Student's *t*-test.

The distribution of α-actin detected by HHF-35 (Dako, USA, 1:2000 dilution) was used as a marker of vascular smooth muscle cells, while the distribution of macrophages was determined using RAM-11 (Dako, USA, 1:2000 dilution). The distributions of MMP-2 and MMP-9 were determined using monoclonal antibodies developed by Daiichi Fine Chemical, Japan (1:1000 dilution). The distribution of MMP-12 was determined using a polyclonal antibody developed by Biogenesis Ltd, UK (1:100 dilution). All sections were processed using a standard avidin-biotin complex method as previously described.[18]

RESULTS

Macroscopic appearance of the aortas: Macroscopic appearance of a rabbit aorta is shown in Figure 1. Although the aortic intimal surfaces in the Chol + ONO

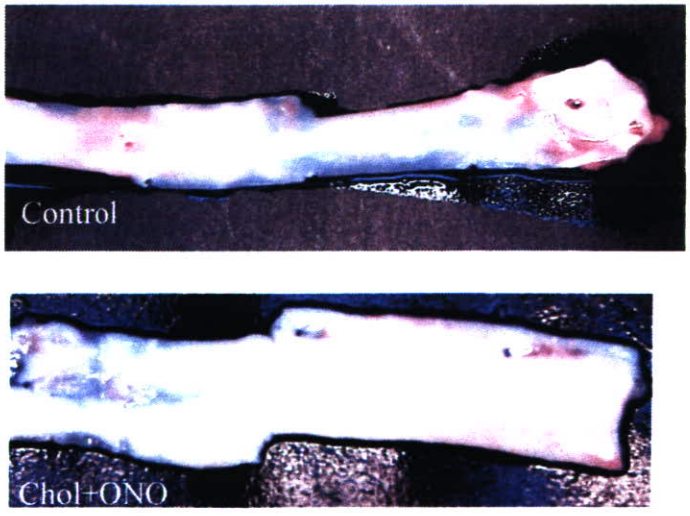

Figure 1. Macroscopic appearance of the aortas.

group had a whitish-yellow appearance, no atherosclerotic plaques were obvious in the aortas of the Chol + ONO group, findings which are in contrast to those of

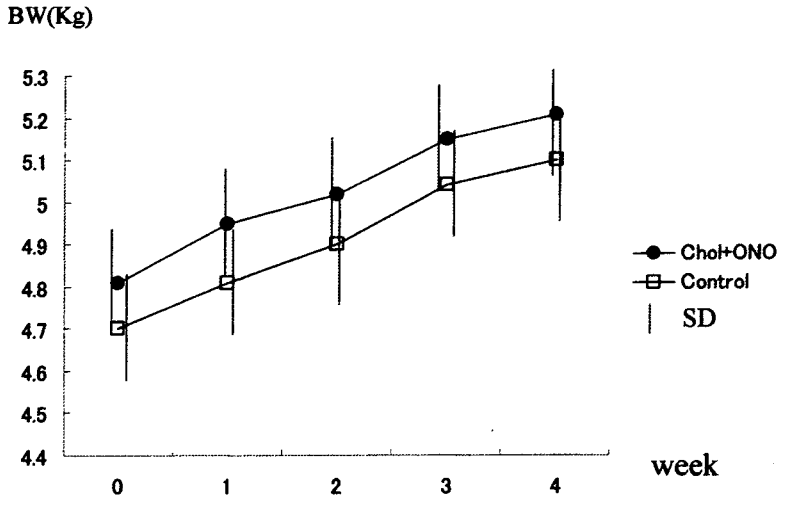

Figure 2. Body weight changes during the study. The mean ± SD of the control and Chol + ONO groups during ONO-4817 administration. The mean body weight of the Chol + ONO group was greater than that of the control group during ONO-4817 administration.

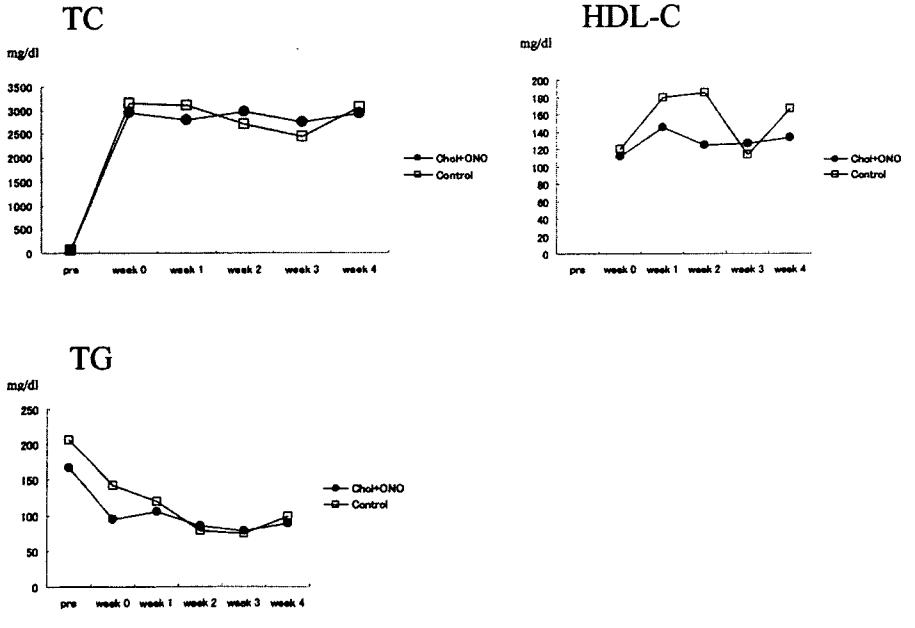

Figure 3. Lipid profile changes. Mean serum total cholesterol (TC), serum triglycerides (TG), and serum HDL-cholesterol (HDL-C) of the control and Chol + ONO groups before (pre) and during ONO-4817 administration (from week 0 to week 4). There were no statistically significant differences in any of the lipid profiles between the 2 groups.

the control group.

Body weight change and lipid profiles of the animals: In both groups, the body weight increased during the study period. Although the average body weight of the Chol + ONO group was greater than that of the control group throughout the treatment period, no statistically significant difference was observed (Figure 2). There were no statistically significant differences in any of the serum lipid profiles between the 2 groups (Figure 3).

Histologic analysis: The histologic appearance of representative aortic crosssections is shown in Figure 4. This section was obtained distal to the branching of the renal arteries (Figure 4). The development of intimal hyperplasia was suppressed in the Chol + ONO group compared with the control group. The ratio of the neointimal lesion to the total cross sectional area (%) was significantly greater in the control group (19.85 ± 9.44%) than in the Chol + ONO group (6.52 ± 1.32%,

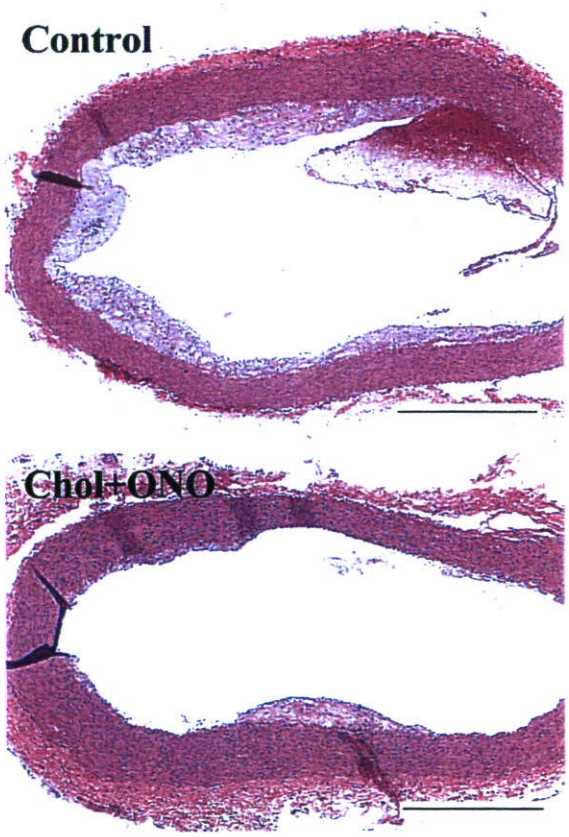

Figure 4. Histological analysis of cross sections of aortas from the control group (Control) and the Chol + ONO group (ONO). Scale bars = 50 μm.

$P < 0.001$). In addition, the histological characteristics of the control group included a more advanced pattern of atherosclerosis than that in the Chol + ONO

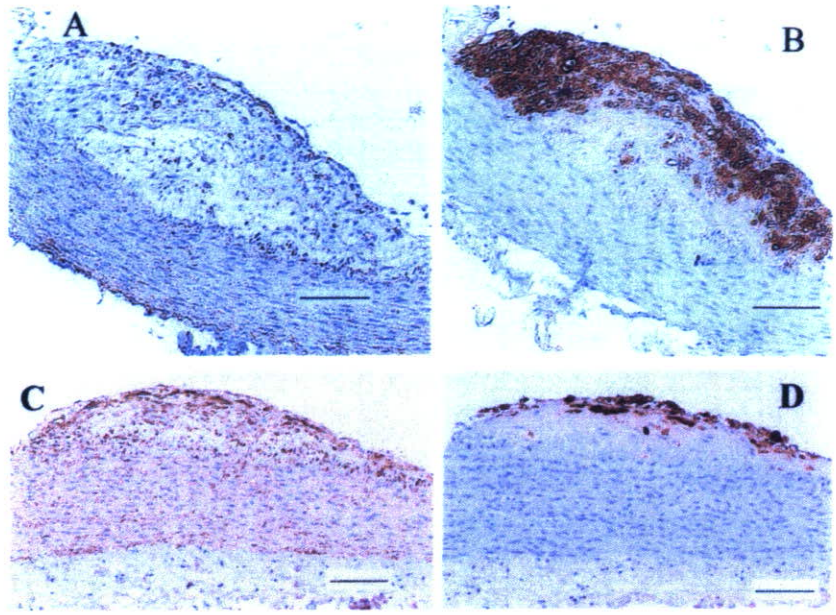

Figure 5. Immunohistochemical analysis of the distribution of smooth muscle cells (α-actin) and macrophages in lesions from both groups. The distribution of α-actin in the control group (panel **A**) did not differ from that in the Chol + ONO group (panel **C**). Although macrophages were diffusely present in the region of atherosclerotic intimal hyperplasia in the control group (panel **B**), macrophages were not as common in the medial part of the region of intimal hyperplasia in the Chol + ONO group (panel **D**). Scale bars = 10 μm.

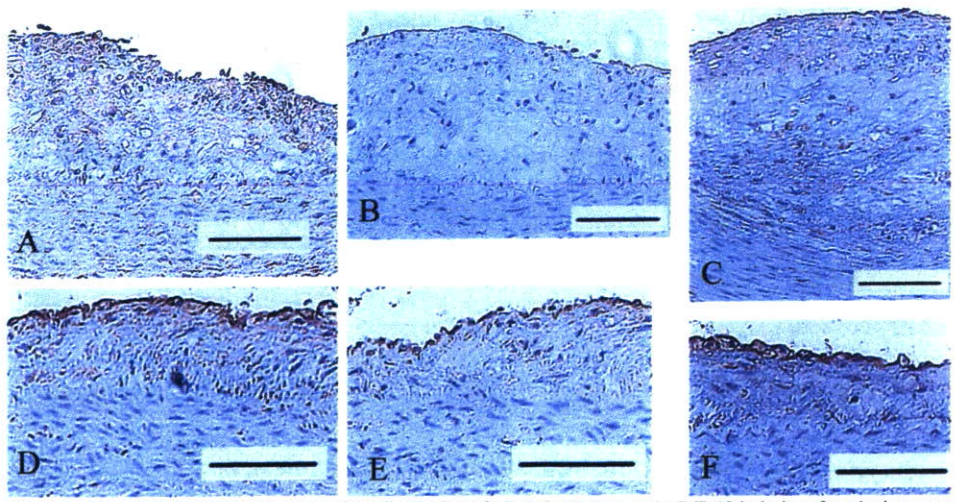

Figure 6. Immunohistochemical analysis of the distribution of MMP-2, MMP-9, and MMP-12 in lesions from both groups. MMP-2 was detected diffusely in the atherosclerotic neointima in the control group (panel **A**). The expression of this MMP was limited to the luminal aspect of the neointima in the Chol + ONO group (panel **D**). Distribution of MMP-9 did not differ between the control group (panel **B**) and the Chol + ONO group (panel **E**). MMP-12 was detected diffusely in the atherosclerotic neointima in the control group (panel **C**). The distribution of this MMP was limited to the luminal aspect of the neointima in the Chol + ONO group (panel **F**). Scale bars = 10 μm.

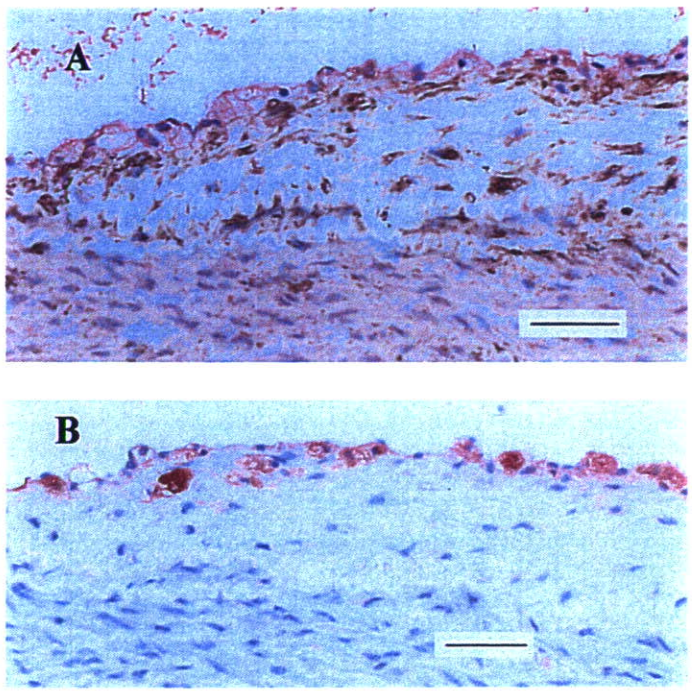

Figure 7. Immunohistochemical double staining for MMP-2 and smooth muscle cells and macrophages in the Chol + ONO group.
Fucsin chromogen (red color) was used to detect MMP-2. Several α-actin negative cells present in the luminal side of the lesion stained positive for MMP-2 (panel **A**). These cells were identified as macrophages (panel **B**). Scale bars = 5 μm.

group. In other words, on both the histological and macroscopic level, the atherosclerotic lesions in the Chol + ONO group were earlier lesions. This suggests that ONO-4817 can inhibit both the development and evolution of atherosclerotic lesions.

Immunohistochemical analysis: The distributions of α-actin (reflecting smooth muscle cells) and of macrophages are shown in Figure 5. Although lesions in the Chol + ONO group were smaller than in the control group, the distribution pattern of α-actin did not differ between the groups. However, the distribution of RAM-11-positive cells (reflecting macrophages) was limited to the luminal aspect of the atherosclerotic neointima in the Chol + ONO group in contrast to the abundant population and the diffuse distribution of these cells in the intimal atherosclerotic lesions in the control group.

The distributions of MMP-2, MMP-9, and MMP-12 are summarized in Figure 6. Because MMP-2 and MMP-9 are reported to be responsible for the migration of smooth muscle cells[19,20] and MMP-12 is reported to be responsible for

plaque instability,[21] we felt that it was important to determine whether ONO-4817 could affect the distribution of these MMPs. Despite the fact that MMP-2 was detected diffusely in the atherosclerotic neointima in the control group (Figure 6A), expression of this MMP was limited in the luminal aspect of the neointima in the Chol + ONO group (Figure 6D). To identify the origin of MMP-2, immunohistochemical double staining for MMP-2 and both α-actin and macrophages was performed (Figure 7). In the Chol + ONO group, cells that were identified as macrophages that did not stain for α-actin were found in the luminal side of the lesion and stained positive for MMP-2.

The distribution of MMP-9 was not different between the control group (Figure 6B) and the Chol + ONO group (Figure 6E).

MMP-12 was detected diffusely in the atherosclerotic neointima in the control group (Figure 6C). However, the distribution of this MMP was limited in the luminal aspect of the neointima in the Chol + ONO group (Figure 6F).

DISCUSSION

Previous reports on the effect of MMPis on atherogenesis have not demonstrated clear histologic effects in animals, including no change in the extent of atherosclerotic intimal hyperplasia.[7,8] We herein have shown that ONO-4817 could inhibit the atherosclerotic neointimal hyperplasia in experimental hyperlipidemic rabbits.

At first, it should be noted that body weight increased during the study in both groups of rabbits. This indicates that ONO-4817 does not affect dietary behavior. Even though there was no statistically significant difference between the body weights of the 2 groups, the higher body weight of the Chol + ONO group during ONO-4817 administration suggests that the inhibitory effect of ONO-4817 on intimal hyperplasia is not dependent on a dietary effect. In addition, we also confirmed that ONO-4817 did not affect the serum lipid profiles of the rabbits. These findings suggest that the inhibitory effect on neointimal hyperplasia by ONO-4817 was independent of the dietary effect and modification of the lipid profile.

The inhibitory effect of ONO-4817 on intimal hyperplasia in this study is thought to be caused by a difference in the distribution of macrophages within the lesion between the control group and the Chol + ONO group. Further studies are therefore needed to determine whether the inhibition of MMP-12 can block the migration of macrophages beneath the arterial intima *in vivo*.

The distribution of smooth muscle cells was not different between the control group and the Chol + ONO group. However, the distribution of MMP-2 was altered in the Chol + ONO group, although no difference was found in MMP-9

Table. Difference in Inhibitory Activities of MMP Inhibitors

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-12	MMP-13
ONO-4817	inactive	0.73	42	2500	1.1	2.1	0.45	1.1
RS-130830	inactive	0.3	9.5	inactive	0.9	0.4	0.7	0.5

ONO-4817, values are mean Ki (nmol/L). RS-1130830, values are mean IC50 (nmol/L).

expression in either group. ONO-4817 has a strong inhibitory effect on MMP-2 and MMP-9.[10] Because these 2 MMPs are thought to be responsible for the migration of smooth muscle cells, blocking the migration of smooth muscle cells by the inhibition of these MMPs could inhibit the formation of atherosclerotic neointima. In fact, we found that the evolution and the prevalence of atherosclerotic intimal hyperplasia were inhibited by ONO-4817. In addition, based on the findings of in vitro studies, ONO-4817 did not inhibit either DNA synthesis in smooth muscle cells or the production of MMP-2 and MMP-9 by smooth muscle cells.[16]

Quantitative analysis should therefore be performed to define the features of the lesions, especially the tissue distribution of collagens and other extracellular matrix components, as well as the lipid content of the lesions. Second, the efficacy of the drug in treating more advanced lesions should be investigated. Furthermore, the effect of the drug on monocyte/macrophage populations, both in the circulation and in the bone marrow, should be studied.

It should be emphasized that there have been no previous reports containing convincing morphologic data demonstrating that MMPis can affect the progression of atherosclerosis.[7,8] Because ONO-4817 may be the first MMPi that is able to suppress the development of intimal hyperplasia induced by intimal injury in hypercholesterolemic hamsters,[16] we have presented data on atherosclerotic intimal hyperplasia in dietary hypercholesteloremic rabbits in this report.

In previous studies[7,8] and a recent study by RS-130830,[9] it was reported that intimal hyperplasia could not be attained by these drugs. The inhibitory activity of RS-130830 and ONO-4817 is summarized in Table.[9,10] The balance of inhibitory activity, not the strength of inhibitory activity, may explain the inhibition of intimal hyperplasia.

To establish the future therapeutic promise for MMPis in the treatment of atherosclerosis, further studies on MMPis in atherosclerosis are therefore eagerly awaited.

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Original Article

Telmisartan Enhances Cholesterol Efflux from THP-1 Macrophages by Activating PPARγ

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Aim: The ATP binding cassette transporters A1 and G1 (ABCA1/G1) and scavenger receptor class B type I (SR-BI) are key molecules in cholesterol efflux and atherogenesis. These genes are regulated by peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor (LXR). Telmisartan is an angiotensin type 1 receptor blocker which has been reported to act as a ligand for PPARγ. We investigated whether PPARγ-activating ARBs affect the expression of these genes and cholesterol efflux from macrophages.

Methods and Results: Telmisartan increased ABCA1, ABCG1 and SR-BI mRNA levels in THP-1 macrophages in a dose- and time-dependent fashion. It also increased their protein levels and enhanced apoA-I- and HDL-mediated cholesterol efflux from macrophages. The knockdown of PPARγ by siRNA abolished the telmisartan-induced expression of these genes. The knockdown of LXRα resulted in the complete and partial abolishment of telmisartan-induced ABCA1 and ABCG1 expression, respectively. We also demonstrated that telmisartan-induced SR-BI expression was dependent on the PPARγ pathway but not on the LXRα pathway. A luciferase assay using an ABCA1 promoter construct showed that telmisartan activated ABCA1 transcription, which was abolished if the LXR binding element was mutated, indicating that increased ABCA1 transcription by telmisartan is LXR-dependent.

Conclusion: Our results showed that telmisartan enhanced both apoA-I- and HDL-mediated cholesterol efflux from macrophages by increasing ABCA1, ABCG1 and SR-BI expression via PPARγ-dependent and LXR-dependent/independent pathways.

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Key words; ARB, PPARγ, LXR

Introduction

The renin-angiotensin system plays an important role not only in the regulation of blood pressure but also in the development of atherosclerosis. After binding to their respective receptors, angiotensin II and insulin reportedly share a signal transduction pathway[1]. On the other hand, angiotensin II inhibits the metabolic actions of insulin via the phosphatidylinositol 3-kinase pathway[2], resulting in insulin resistance. Angiotensin type 1 receptor (AT_1R) blockers (ARBs) and angiotensin-converting enzyme inhibitors, which function as antihypertensive agents, have been reported to prevent atherosclerosis in a study using monkeys[3], improve insulin resistance[4], and induce a favorable lipid profile[5,6]. Besides their metabolic effect on insulin signaling, telmisartan[7,8] and irbesartan[7], two ARBs, have been found to activate peroxisome proliferator-activated receptor γ (PPARγ) in cells derived from rodents and this is independent of their AT_1R blocking actions.

Recent evidence shows that a transcriptional cas-

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cade in the PPARγ and liver X receptor (LXR) pathways is important for maintaining cellular cholesterol homeostasis in macrophages[9, 10] and activating PPARγ results in increased LXRα expression, which in turn transactivates the target genes[9]. LXR and/or PPARγ reportedly up-regulated the ATP binding cassette transporters (ABC) A1[9, 11], and ABCG1[12, 13] and scavenger receptor class B type I (SR-BI)[14], all of which facilitate cellular cholesterol efflux[12, 15-18]. The deletion of PPARγ[9, 19], LXR[20], ABCA1[21], ABCG1[22], and SR-BI[23] in macrophages reportedly accelerates the development of atherosclerosis and treatment with LXR[24] and PPARγ[25] ligands inhibits its development; however, it is still not clear whether PPARγ-activating ARBs affect the expression of these genes and cholesterol efflux from macrophages, and regulate PPARγ-inducible genes in human cells[26].

In this study, we demonstrated that telmisartan increased ABCA1, ABCG1 and SR-BI expression and enhanced apolipoprotein (apo) A-I- and high density lipoprotein (HDL)-mediated cholesterol efflux from THP-1 macrophages. The telmisartan-induced expression of these genes was regulated by PPARγ activation in both an LXRα-dependent and independent manner.

Methods

Materials

Telmisartan (Boehringer Ingelheim, Ingelheim, Germany), pioglitazone (Takeda Chemical Industries, Osaka, Japan), losartan (Cayman Chemical, Ann Arbor, MI, USA) and 22(R)-hydroxy cholesterol (22HC; Sigma, St. Louis, MO, USA) were dissolved in dimethylsulfoxide (Sigma). Human apo A-I was purchased from Sigma, and human HDL_2 and HDL_3 subfractions from Acris Antibodies GmbH (Hiddenhausen, Germany).

Cell Culture

THP-1 cells (Riken Cell Bank, Tokyo, Japan) were maintained in RPMI 1640 (Sigma) containing 10% fetal bovine serum (FBS). The differentiation of THP-1 monocytes into macrophages was induced in the presence of 200 nM of phorbol 12-myristate 13-acetate (PMA; Wako, Tokyo, Japan) for 72 hr. Human peripheral blood monocytes were isolated using the method of Fogelman *et al.*[27] with Ficoll/Hypaque gradient centrifugation. The mononuclear cells thus obtained were re-suspended in RPMI 1640 (Sigma) supplemented with 20% autologous serum, plated onto serum-treated 10-cm dishes and incubated for 2 hr. Non-adherent cells were removed by washing three times with phosphate-buffered saline (PBS), and adherent cells were then detached by incubation in PBS containing 5% autologous serum and 0.02% EDTA at 4℃ for 30 min. The adherent cells were then washed extensively and re-suspended in RPMI 1640 supplemented with 5% autologous serum. They were then plated on 10-cm dishes and incubated for 10 days so that they would differentiate into macrophages.

Real-time Quantitative RT-PCR

At the indicated hours after treatment with telmisartan and the other compounds, total RNA was extracted from the cells, and first-strand cDNA was synthesized from total RNA (250 ng) by placing in a Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA)[28]. Quantitative PCR was performed using a Perkin-Elmer 7900 PCR machine, TaqMan PCR master mix and FAM-labeled TaqMan probes (Assays-on-Demand, Applied Biosystems) for human ABCA1, ABCG1, SR-BI, PPARγ, LXRα, LXRβ, apoE, CD11b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression data were normalized for GAPDH levels.

Western Blot Analyses

Cells were harvested and protein extracts prepared as previously described[28]. They were then subjected to Western blot analyses (10% SDS-PAGE; 30 μg protein per lane) using rabbit anti-ABCA1- (Novus Biologicals, Littleton, CO, USA), ABCG1- (Novus Biologicals), SR-BI- (Novus Biologicals), mouse anti-LXRα- (PPMX, Tokyo, Japan) and β-actin (Santa Cruz, Santa Cruz, CA, USA)-specific antibodies. The proteins were visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; Amersham Biosciences, Foster City, CA, USA).

Determination of Cholesterol Efflux

Cholesterol efflux experiments were performed as previously described[28]. After 72 hr of THP-1 monocyte differentiation into macrophages, the macrophages so produced were labeled with [^{3}H]cholesterol (1.0 μCi/mL) in RPMI 1640 containing 0.2% bovine serum albumin (BSA) for 20 hr. The cells were washed twice with PBS and incubated for 24 hr in RPMI 1640 containing 0.2% BSA plus telmisartan (20 μM), pioglitazone (10 μM) or the vehicle. The macrophages were again washed with PBS and incubated in RPMI 1640 containing 0.2% BSA in the presence and absence of apoA-I (10 μg/mL), HDL_2 (50 μg/mL) or HDL_3 (50 μg/mL), for 24 hr. The percentage cholesterol efflux was calculated by dividing media-derived radioactivity by the sum of the radioactivity in the media and cells.

PPARγ Activity Assay

PPARγ transcription factor activity was assayed using an enzyme-linked immunosorbent assay-based PPARγ transcription factor activity assay kit to detect and confirm transcription PPARγ factor activation (Active Motif, Carlsbad, CA, USA). THP-1 macrophages were treated with telmisartan, pioglitazone or vehicle for 2 hr. The cells were then rinsed, and nuclear protein was extracted according to the manufacturer's instructions. Nuclear extracts were added to a 96-well plate that had been immobilized by an oligonucleotide containing peroxisome proliferator-response elements (5'-AACTAGGTCAAAGGTCA-3'). After 1 hr, the wells were incubated with diluted primary PPARγ antibody to recognize the accessible epitope on PPARγ protein upon DNA binding. The horseradish peroxidase-conjugated secondary antibody was added and incubation conducted for 1 hr. At the end, the reaction was stopped, and absorbance was read at 450 nm on a spectrophotometer. This assay is specific for PPARγ activation, and there is no cross-reaction with PPARα or PPARβ.

siRNA-mediated Macrophage RNA Interference

Human PPARγ- and LXRα-specific small interfering RNA (siRNA) and scrambled control RNA oligonucleotides were purchased from Ambion Inc. (Austin, TX, USA). The transfection of siRNA was performed using TransIT-TKO Transfection Reagent according to the manufacturer's instructions (Mirus Bio Corporation, Madison, WI, USA). Briefly, 40 nM of scrambled control RNA oligonucleotide or either PPARγ-siRNA or LXRα-siRNA were added to THP-1 cells 48 hr after treatment with PMA for differentiation. The cells were incubated for a further 24 hr, washed and then the indicated doses of telmisartan and pioglitazone were added. The cells were harvested 24 hr later and mRNA levels determined using RT-PCR. The oligonucleotide sequences used to construct siRNA used in this study were: 5'-GGAUGCAAGGGUUUCUUCCtt-3' and 5'-GGAAGAAACCCUUGCAUCCtt-3' for PPARγ (PPARγ-siRNA); and 5'-GGAGUGUGUCCUGUCAGAAtt-3' and 5'-UUCUGACAGGACACACUCCtc-3' for LXRα (LXRα-siRNA).

Construction of Luciferase Reporter Plasmids, DNA Transfection and Luciferase Assays

Luciferase reporter plasmids, ABCA1-Luc and ABCA1-Luc DR4mut, which respectively contain the human ABCA1 promoter region spanning −940 to +110 bp with or without mutations in direct repeat 4 (DR4), were constructed as previously described[28]. Forty-eight hr after treatment with PMA, THP-1 cells were transfected with 1.8 μg of ABCA1-Luc or ABCA1-Luc DR4mut and 0.1 μg of phRL-TK (Promega) per well using the TransIT-TKO Transfection Reagent. Twenty-four hr after transfection, the media were replaced with RPMI 1640 containing 5% FBS, with or without the indicated doses of telmisartan, pioglitazone or 22HC, and incubated for an additional 24 hr. Luciferase assays were performed as previously described[28].

Statistical Analysis

ANOVA and the Mann-Whitney U test were performed for statistical analysis as appropriate. Statistical significance was designated at $p<0.05$. Values are expressed as the mean ± SEM.

Results

Telmisartan Induces ABCA1, ABCG1, SR-BI and LXRα Gene Expression

Twenty-four hr after treatment with telmisartan, ABCA1, ABCG1, SR-BI and LXRα mRNA levels in THP-1 macrophages had increased in a dose-dependent manner (1-20 μM, C_{max}: 1.8 ± 1.1 μM) after administration of telmisartan 80 mg to healthy subjects[29], and similar changes were noted in response to treatment with the PPARγ ligand, pioglitazone (**Fig. 1A**). The other ARB, losartan, had no significant effect on expression of these genes, suggesting that telmisartan increased their expression independently of its AT_1R blocking action (**Fig. 1A**). **Fig. 1B** shows that telmisartan produced a marked increase in SR-BI and LXRα mRNA levels up to 8 hr after treatment, but the real increases in ABCA1 and ABCG1 mRNA levels came after 8 hr and continued after 16 hr. In contrast, there was a decrease in PPARγ mRNA levels up to 16 hr which continued to be low thereafter.

We also observed that 10 μM of telmisartan significantly increased ABCA1, ABCG1, SR-BI and LXRα mRNA levels in human monocyte-derived macrophages (**Fig. 2**), and that telmisartan and pioglitazone increased PPARγ transcription factor activity, using an enzyme-linked immunosorbent assay-based PPARγ transcription factor activity assay kit (**Fig. 3**). There was no change in LXRβ mRNA levels after treatment with telmisartan or the other compounds (data not shown).

Telmisartan Increases ABCA1, ABCG1 and SR-BI Protein Levels and Cholesterol Efflux from THP-1 Macrophages

Having established that telmisartan increased ABCA1, ABCG1, SR-BI and LXRα mRNA levels, we

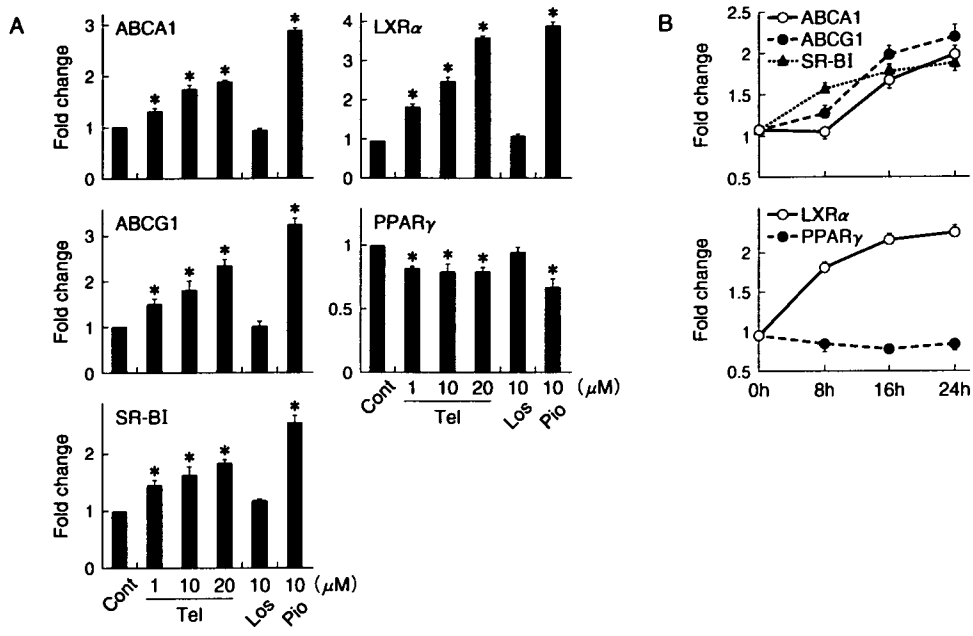

Fig. 1. Telmisartan increases ABCA1, ABCG1, SR-BI and LXRα, and attenuates PPARγ gene expression in THP-1 macrophages.

THP-1 macrophages were treated with the vehicle (Cont) or the indicated concentrations of telmisartan (Tel), losartan (Los), or pioglitazone (Pio) for 24 hr (A) or indicated hours (B). Real-time quantitative RT-PCR was performed as described in Methods. The mRNA levels of each gene were standardized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The results from 3 separately performed experiments are expressed relative to the controls and presented as the mean ± SEM. $^*p<0.05$ vs control.

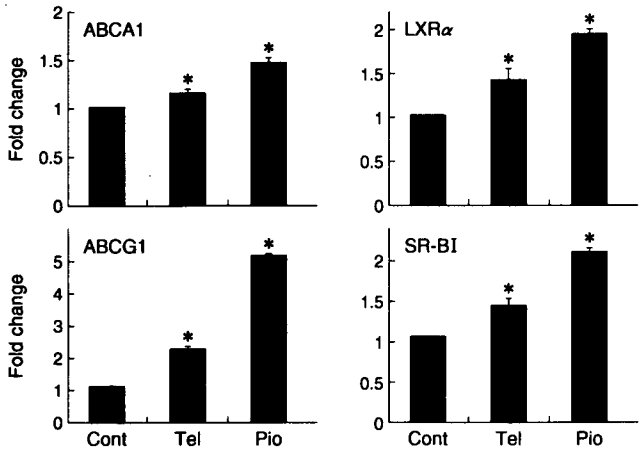

Fig. 2. Effects of telmisartan and pioglitazone on ABCA1/G1, LXRα and SR-BI mRNA levels in human monocyte derived macrophages (HMDM).

HMDM were treated with 20 μM of telmisartan, 10 μM of pioglitazone or vehicle for 24 hr. Total RNA was extracted and real-time quantitative RT-PCR was performed as described in Materials and Methods to determine ABCA1 mRNA expression levels. The relative ABCA1 mRNA abundance was calculated by dividing the values for the expression levels of ABCA1 by those for GAPDH. The results for 3 samples are presented as the mean ± SE. $^*p<0.05$ versus control. *P* values were calculated using Mann-Whitney's U test.

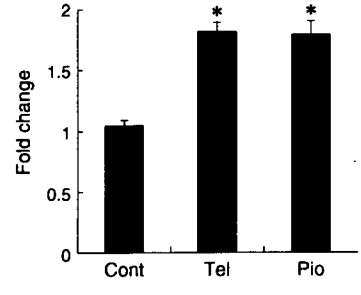

Fig. 3. Telmisartan enhances PPARγ transcriptional activity.

THP-1 macrophages were treated with 20 μM telmisartan, 10 μM pioglitazone or vehicle for 24 hr. The preparation of nuclear extracts and the measurement of PPARγ transcription activity were performed as described in Materials and Methods. The results for 3 samples are presented as the mean ± SE. $^*p<0.05$ versus control. *P* values were calculated using Mann-Whitney's U test.

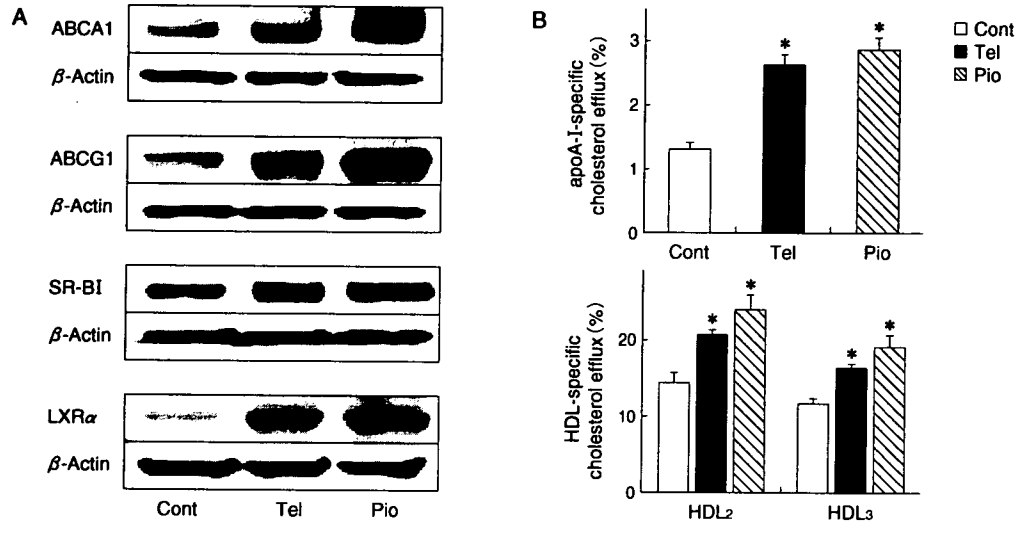

Fig. 4. Effects of telmisartan on ABCA1, ABCG1, SR-BI and LXRα protein levels and cholesterol efflux from THP-1 macrophages.

A, THP-1 macrophages were lysed and subjected to Western blot analysis as described in Methods after 24 hr (SR-BI and LXRα) or 48 hr (ABCA1 and ABCG1) of treatment with vehicle (Cont), 20 μM of telmisartan (Tel), or 10 μM of pioglitazone (Pio). B, Cholesterol efflux from THP-1 macrophages mediated by apoA-I or HDL was determined as described in Methods. After cholesterol labeling, 20 μM of Tel or vehicle (Cont) were added to the cultures which were then incubated in the presence of 20 μg/mL of human apoA-I, 50 μg/mL of HDL_2 or HDL_3 for 24 hr. The results for 4 samples are presented as the mean ± SEM. $^*p < 0.05$ vs control.

examined its effect on the protein levels of these molecules and cholesterol efflux from THP-1 macrophages in comparison with pioglitazone. Both telmisartan and pioglitazone increased ABCA1, ABCG1, SR-BI and LXRα protein levels and this was consistent with the increases in mRNA levels observed (**Fig. 4A**). Mirroring the increases in the protein levels of ABCA1, ABCG1 and SR-BI they brought about, telmisartan and pioglitazone significantly increased the cholesterol efflux mediated by both apoA-I and HDL (**Fig. 4B**).

PPARγ- and LXRα- siRNA Attenuate the Stimulatory Effect of Telmisartan on ABCA1, ABCG1 and SR-BI mRNA Levels

We used the siRNA approach to determine whether the telmisartan-induced expression of ABCA1, ABCG1 and SR-BI is dependent on PPARγ and/or LXRα. PPARγ- and LXRα-siRNA suppressed PPARγ and LXRα mRNA levels by up to 75% and up to 91%, respectively (**Fig. 5A** and **5C**). In these experiments, transient transfection of THP-1 macrophages with PPARγ-siRNA substantially abolished telmisartan-mediated induction of ABCA1, ABCG1, SR-BI and LXRα expression (**Fig. 5B**). Nonsilencing, scrambled siRNA had no effect. LXRα-siRNA also abolished the increase in ABCA1 mRNA levels brought about by telmisartan (**Fig. 5D**); however, it only reduced telmisartan-induced ABCG1 expression by up to 20% and had no effect on the increase in SR-BI mRNA levels induced by telmisartan (**Fig. 5D**). These findings suggest that telmisartan-induced ABCA1 and ABCG1 expression is mediated by PPARγ, and that the effect of telmisartan on ABCA1 and ABCG1 is completely and partially dependent on LXRα, respectively. Furthermore, it seems that the increase in SR-BI expression due to telmisartan is dependent on PPARγ, but not LXRα.

Increase in ABCA1 Gene Expression Induced by Telmisartan is Associated with LXR-Dependent Transcriptional Activation

To investigate the effect of telmisartan on ABCA1 promoter activity, ABCA1-Luc was transfected into THP-1 macrophages and a luciferase assay was performed (**Fig. 6**) in experiments in which telmisartan, pioglitazone and an LXR ligand, 22HC, were added. Telmisartan increased ABCA1 promoter activity in pace with the increase in mRNA levels it brought about (**Fig. 6B**). Pioglitazone and 22HC also enhanced transcription of the ABCA1 reporter gene.

It has been reported that LXRs form heterodimers with retinoid X receptor (RXR) and bind to conserved consensus cis-element, DR4, in the ABCA1 promoter region, resulting in the activation of transcrip-

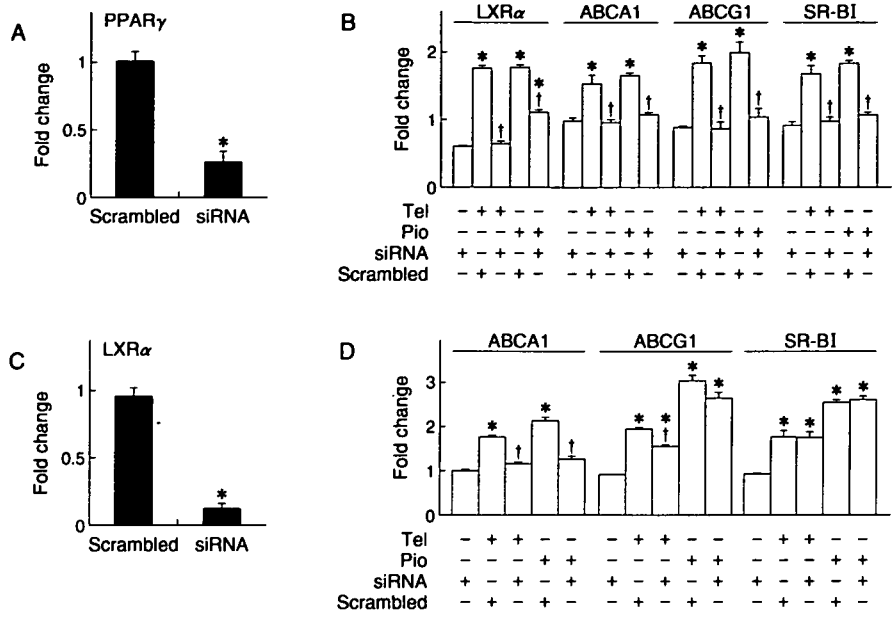

Fig. 5. Effects of PPARγ- and LXRα-siRNA on macrophage gene expression induced by telmisartan and pioglitazone.

Incubation was conducted with scrambled siRNA, PPARγ-siRNA (A, B) or LXRα-siRNA (C, D) during THP-1 differentiation into macrophages as described in Methods. Twenty-four hr after treatment with siRNAs, cells were harvested and the mRNA levels of PPARγ (A) and LXRα (C) were measured using RT-PCR. Further incubation was conducted with 20 μM of Tel, 10 μM of Pio or the vehicle, and then cells were harvested for mRNA measurement (B, D). Expression levels were standardized for GAPDH levels. The average expression level obtained for cells treated with scrambled siRNA and the vehicle is presented as the control, and the results of 3 separately performed experiments are expressed relative to the control and presented as the mean ± SEM. $^{*}p<0.05$ vs. control; $^{\dagger}p<0.05$ vs. scramble siRNA in the presence of identical compounds.

tion[11]. When the DR4 mutant-containing reporter vector (ABCA1-Luc DR4mut, **Fig. 6A**) was used, telmisartan had no effect on ABCA1 promoter activity, and neither did pioglitazone or 22HC (**Fig. 6B**), indicating that telmisartan-activated ABCA1 transcription is dependent on LXR.

Discussion

Our observations suggest that the effect of telmisartan on the expression of ABCA1, ABCG1, SR-BI and LXRα is mediated by PPARγ activation, and not by AT1R blockade. Schupp *et al.*[30] observed that typical PPARγ ligands and the individual PPARγ-activating ARBs, telmisartan and irbesartan, brought about distinct conformational changes in PPARγ, which were associated with selective cofactor recruitment and a distinctive gene expression profile, in murine adipocytes. They also demonstrated that pioglitazone and telmisartan differentially regulate ABCA1 (do not change and decrease, respectively) and ABCG1 (decrease and do not change, respectively) expressions in 3T3-L1 adipocytes using DNA microarrays. The discrepancy between their observations and our findings could be due to species- and/or cell-specific regulation of these genes including distinct cofactors involved in the transcriptional machinery. The present study demonstrated telmisartan's PPARγ-activating effects in human cells for the first time[26], and that telmisartan stimulates the PPARγ-LXRα pathway, resulting in ABCA1/G1 activation, effects which have not been observed in murine adipocytes[30].

The experiments using siRNAs with the objective of suppressing PPARγ or LXRα expression indicated that the increases in ABCA1 mRNA levels due to telmisartan and pioglitazone were almost completely mediated via the PPARγ-LXRα pathway. Also, in the assays to measure promoter activity, we found that the increase in ABCA1 transcriptional activity due to telmisartan and pioglitazone was not observed when DR4 was mutated. Furthermore, a recent study[13] noted that a PPARγ agonist had a stimulatory effect on ABCA1

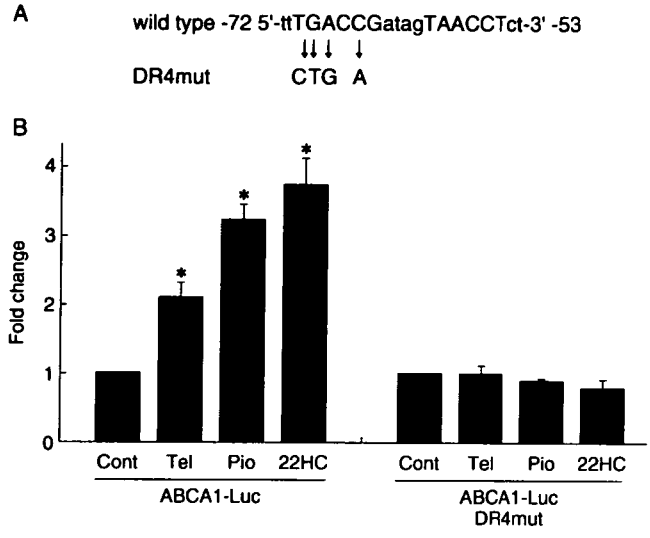

Fig. 6. Telmisartan activates ABCA1 transcription via LXR in THP-1 macrophages.

A, Mutated nucleotides in DR4mut construct. Numbers indicate positions relative to the site of starting transcription. Upper case characters indicate half-sites of the DR4 element. B, After transfection with ABCA1-Luc or ABCA1-Luc DR4mut, the macrophages were treated with vehicle (Cont), 20 μM of telmisartan (Tel), 10 μM of pioglitazone (Pio), or 10 μM of 22(R)-hydroxycholesterol (22HC) as described in Methods. Twenty-four hr after the treatments, the cells were lysed and a luciferase assay performed. The results from 3 separately performed experiments are expressed relative to the controls and presented as the mean ± SEM. $^*p<0.05$ vs control.

expression in wild-type macrophages but not on LXRα/LXRβ double knockout macrophages. These findings indicate that activated PPARγ increases ABCA1 mRNA levels at the transcriptional level in an LXR-dependent manner.

After treatment with LXRα-siRNA, we noted that telmisartan-induced ABCA1 expression was substantially blocked whereas ABCG1 expression was only partially blocked. In contrast, ABCG1 expression was completely suppressed by PPARγ-siRNA. In this regard, Li *et al.*[13] reported no difference in the stimulatory effect of PPARγ agonists on ABCG1 expression between wild-type and LXRα/LXRβ double knockout macrophages, suggesting that the activation of PPARγ-induced ABCG1 expression is independent of LXR. On the other hand, Laffitte *et al.*[31] reported that LXR agonist-induced ABCA1 and ABCG1 expressions in macrophages were completely abolished by the deletion of both LXRα and LXRβ. Other studies have shown that the ABCG1 gene is transcriptionally activated by activated LXR through its binding elements[12]. Taken together, the above observations suggest that ABCG1 is regulated via PPARγ, both in an LXRα-dependent and -independent manner, and the LXRα-independent effect of PPARγ activation on ABCG1 expression could overwhelm the inhibitory effect of LXRα knockdown on ABCG1. As it is still not clear whether this LXR-independent effect is mediated by PPARγ directly or by other pathways affected by PPARγ, we are currently conducting further studies to determine whether ABCG1 is transcriptionally regulated by PPARγ.

The present study suggests that both pioglitazone- and telmisartan-mediated induction of SR-B1 mRNA expression are independent of the LXR pathway. We also found that an LXR agonist brought about no changes in SR-B1 mRNA levels in macrophages (data not shown). Recently, other researchers have demonstrated that SR-B1 expression in atherosclerotic lesion macrophages is regulated by PPARγ[14] and activated PPARγ transcriptionally stimulates hepatic SR-B1 expression[32]. The SR-B1 expression in macrophages induced by pioglitazone and telmisartan in our study could be due to a similar mechanism.

The changes in ABCA1, ABCG1, SR-B1 and LXRα levels with time following treatment with telmisartan suggest that SR-B1 is directly up-regulated by telmisartan-mediated PPARγ and, in the case of ABCA1, telmisartan first activates LXRα, which then induces ABCA1 transcription, consistent with the results obtained in siRNA experiments. The results of these experiments indicate that the newly produced proteins induced by PPARγ and/or activated PPARγ could be involved to a major extent in increased ABCG1 expression due to telmisartan, and those induced by LXRα might only be partly involved.

Interestingly, telmisartan and pioglitazone down-regulated PPARγ mRNA levels in THP-1 macrophages in our study. These observations are similar to the findings of Schupp *et al.*[30] who showed that PPARγ mRNA levels were down-regulated after incubation with telmisartan and pioglitazone in murine adipocytes. Since the mechanism for this negative regulation of PPARγ expression by its ligands is still unclear, further study is necessary.

Our data show that telmisartan enhanced both apoA-I- and HDL-mediated cholesterol efflux, and increased ABCA1, ABCG1 and SR-B1 protein levels, which was consistent with changes in mRNA levels. It has been reported that lipid-poor apoA-I contributes to ABCA1-mediated cholesterol efflux from cells, but not HDL[15, 16]. On the other hand, ABCG1 has been reported to promote cholesterol efflux to HDL, but not to apoA-I[16, 17]. SR-B1 is also known to promote HDL-mediated cellular cholesterol efflux[18]. Further, recent research has demonstrated that the deletion of ABCA1[21], ABCG1[22], and SR-B1[23] in macrophages

results in increased atherogenesis. Telmisartan has already been observed to have an anti-atherosclerotic effect in a study using monkeys[3] and all of the above evidence suggests that in this effect, there could be a contribution from its ability to enhance cholesterol efflux from macrophages. Regarding ABCG1, recent studies have reported a decrease in atherosclerosis in mice transplanted with ABCG1-knockout bone marrow[33, 34]; however, no study has demonstrated the effect of ABCG1 overexpression in macrophages on the development of atherosclerosis. Therefore, further studies are needed to assess whether increased cholesterol efflux from macrophages due to telmisartan-induced ABCG1 expression contributes to the development of atherosclerosis.

There is accumulating evidence showing that PPARγ-activating ARBs bring about favorable changes in glucose and lipid metabolism. The combination of this effect and their antihypertensive action could help attenuate the development of atherosclerotic diseases. The present study demonstrates that PPARγ activation by telmisartan is part of a novel pathway having possibilities for atherogenesis prevention, in which cholesterol efflux from macrophages is increased, and ABCA1, ABCG1 and SR-BI expressions are enhanced. Clinical studies are now needed to investigate whether PPARγ-activating ARBs would be superior to non-PPARγ-activating ARBs in terms of preventing atherosclerotic diseases.

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Case Report

Large Right Coronary Artery to Left Ventricle Fistula

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Abstract

Coronary artery fistulas are uncommon entities with communication between the coronary artery and cardiac structure. We encounter a 70-year-old, asymptomatic woman with a large right coronary artery to left ventricle fistula. Non-invasive echocardiogram was helpful in detecting an enlarged right coronary that drained from the aorta into the left ventricle. Clinical importance of coronary artery fistula is due to an increased risk of heart failure, myocardial ischemia, and rupture. Treatment of asymptomatic patients without significant shunting is controversial. We decided to follow-up on her medically without surgical closure of the fistula because her hemodynamics are stable.
Echocardiogram is very useful to diagnose and evaluate these diseases. (J Echocardiogr 2007; 5: 58-60)

Key words: right coronary artery to left ventricular fistula

Case Report

A 70-year-old, asymptomatic woman was referred to our department for further evaluation of a grade three diastolic murmur at the right sternal border in the third intercostal space and slight cardiac enlargement on chest X-ray. The electrocardiogram showed slightly high voltages. We could detect the whole length of an enlarged right coronary artery draining from the aorta into the posterior wall of her left ventricle, with blood flow detected mainly in diastolic color flow image component by echocardiogram (Figure. 1). A calculated ejection fraction was 65% with a left ventricle diameter of 53mm in diastole and 37mm in systole. We diagnosed her as having coronary artery fistula. Multidetection computed tomography (MDCT) and coronary angiogram supported the diagnosis (Figure. 2). Aortogram showed that shunt flow had the same

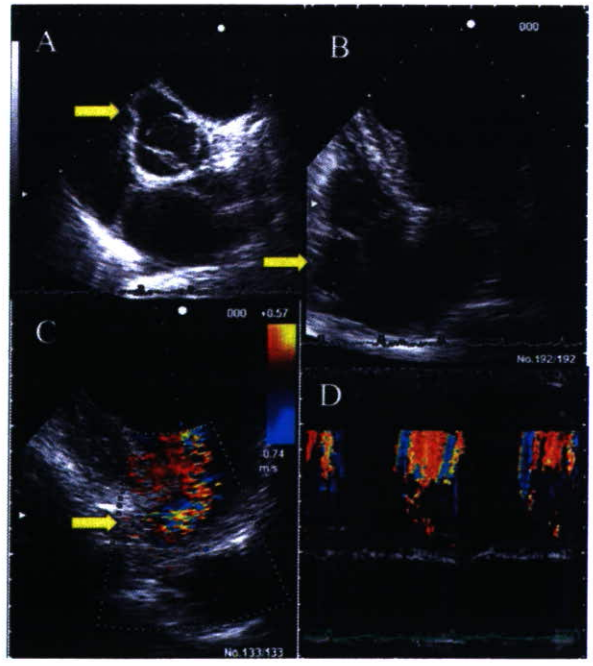

Fig. 1. Echocardiogram
A. short axis view of aortic valve. The arrow shows large right coronary artery drain from aorta. B. The arrow shows that max major axis of fistula is 2.4 cm. C, D 3-chamber view of diastolic phase. The blood flow is mainly displayed as diastolic color flow image component.

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