의학박사 학위논문

Combined application of rapamycin and atorvastatin attenuates atherosclerosis and improves lipid metabolism in apolipoprotein E-deficient mice with chronic kidney disease

신부전 유도 아포지단백질 E결핍 마우스에서 라파마이신과 아토바스타틴의 병용 투여 후 동맥경화 및 지질대사 개선 효과

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서울대학교 대학원

의학과 외과학 전공

안 상 현

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이 논문을 의학박사 학위논문으로 제출함

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안상현

안상현의 의학박사 학위논문을 인준함

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Abstract

Background: Atherosclerosis arising from the pro-inflammatory conditions associated with chronic kidney disease (CKD) increases major cardiovascular morbidity and mortality. rapamycin is known to inhibit atherosclerosis under CKD and non-CKD conditions, but it can cause dyslipidemia; thus, the co-application of lipid-lowering agents is recommended. Atorvastatin has been widely used to reduce serum lipids levels, but its synergic effect with rapamycin in CKD remains unclear. Here, we analyzed the effect of their combined treatment on atherosclerosis stimulated by CKD in apolipoprotein E-deficient (ApoE-/-) mice.

Methods: The mice were randomly assigned to five groups, including one group with normal renal function (sham-operated) and the others with surgically induced CKD (by 5/6 nephrectomy) (vehicle vs. atorvastatin vs. rapamycin vs. rapamycin + atorvastatin). atorvastatin (10 mg/kg) and rapamycin (0.5 mg/kg) were administered by daily oral gavage for 10 weeks. Aorta and aortic sinus were stained with Oil Red O to compare the size of atherosclerotic lesions. The expression levels of lipid metabolism-related genes and proteins in the livers was evaluated by qRT-PCR and Western blot. The expression level of pro/anti-inflammatory cytokines in aorta and spleen was measured and compared using qRT-PCR.

Results: Oil Red O staining revealed that treatment with rapamycin and

rapamycin + atorvastatin, but not atorvastatin alone, significantly decreased the atherosclerotic lesions in the aorta and aortic sinus, compared to those seen in the control (CKD) group. The co-administration of rapamycin and atorvastatin improved the serum lipid profile and enhanced the liver expression levels of genes involved in cholesterol transport (ABCG5), bile acid biosynthesis (CYP7A1), and lipid metabolism (PPARɣ, ApoA1). The CKD group showed increased levels of various genes encoding atherosclerosis-promoting cytokines in the spleen (TNF-α, IL-6 and IL-1β) and aorta (TNF-α and IL-4), and these increases were attenuated by rapamycin treatment. atorvastatin and rapamycin + atorvastatin decreased the levels of TNF-α and IL-1β in the spleen, but not in the aorta.

Conclusion: These results indicate that, in CKD-induced ApoE-/- mice, rapamycin significantly reduces the development of atherosclerosis by regulating the expression of inflammatory cytokines and the co-application of atorvastatin improves lipid metabolism.

Keywords: Chronic kidney disease, atherosclerosis, rapamycin, atorvastatin

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Chapter 1. Introduction

1.1 Chronic Kidney Disease Accelerates Atherosclerosis Causing Cardiovascular Diseases

Atherosclerosis and arterial calcification are more frequently developed and severe in patients with chronic kidney disease (CKD) than the general population.(1) Uremia in CKD patients precipitates oxidative stress and inflammation on the arteries and accelerates plaque formation, which is called accelerated atherosclerosis.(2, 3) As a result, CKD patients are associated with increased morbidity and mortality due to cardiovascular diseases.(4)

1.2 Mechanism of Accelerated Atherosclerosis in CKD

The pathogenic mechanism of accelerated atherosclerosis in CKD can be explained by an imbalance of electrolytes such as calcium (Ca) and phosphate (P), and loss of vascular smooth muscle cell (VSMC) function.(5) Also, since the kidney is the main organ of cytokine removal, CKD patients have cytokine dysregulation and persistent inflammation that can stimulate vascular cell senescence.(6-11) Another cause for atherosclerosis could be an alteration of cholesterol homeostasis, including increased low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL).(12) An immune-metabolic imbalance could be a culprit of atherosclerosis.(13, 14)

1.3 Atorvastatin Attenuates Atherosclerosis

Several studies reported the inhibitory effect of statins, including atorvastatin against atherosclerosis in non-CKD mouse models. atorvastatin therapy has been known to have an anti-inflammatory effect by inhibiting the production of tumor necrosis factor (TNF)-α and protecting VSMCs against TGF-β1 simulation.(15-17) Also, atorvastatin promotes autophagy via β-catenin pathway and serum osteoprotegerin (OPG) and suppresses circulating osteoprogenitor cells and receptor activator of nuclear factor kappa-B ligand (RANKL) expression, which withhold degeneration of VSMCs.(18) However, the effects of atorvastatin on CKD is doubtful and may play a limited role in inhibiting atherosclerosis.(19) In a similar lines, atorvastatin has not shown the difference in the C-reactive protein level and in survival in a human study.(20)

1.4 Protective Effect of mTOR Inhibitor (Rapamycin) Against Atherosclerosis

Zhai and Xiong et al. demonstrated that atherosclerosis was associated with PI3K/Akt/ mTOR signaling pathway and mTOR/PRKAA/AMPK signaling.(21, 22) In the same way, Atherosclerosis in apolipoprotein E knockout mice and in vascular smooth muscle cells is promoted by increased activity of mTOR complex1.(23)

Rapamycin, an mTOR inhibitor, is an immunosuppressive agent that suppresses the development of atherosclerosis and arterial calcification.(24, 25) Amelioration of atherosclerosis by rapamycin attained through chemokine downregulation and Klotho upregulation.(13, 25) Also, rapamycin upregulated autophagic degradation of ox-LDL in human umbilical vein endothelial cells.(26)

Despite this, rapamycin is known to cause dyslipidemia and it is therefore recommended for use with lipid-lowering agents.(27)

1.5 Combination of Atorvastatin and Rapamycin

Combination of statin is expected to control dyslipidemia caused by rapamycin and directly suppress mTOR complex1.(28) A study demonstrated that the combination of rapamycin and atorvastatin in vascular endothelium synergistically decreased complement-mediated injury via PKCα-, AMPK-, and CREB-dependent pathway.(29) However, there is little direct research on the synergic effect of rapamycin and atorvastatin for inhibition of atherosclerosis, especially in CKD.

1.6 Purpose of the Study

To understand the mechanism of CKD-accelerated atherosclerosis in depth, in vivo CKD models are required. Although several mouse models have been developed for investigating the mechanisms of atherosclerosis caused by CKD,

limitations exist because the genetic manipulations or inducing methods are associated with various degrees of renal failure.(30) In this study, we established a distinct CKD mouse model promoting atherosclerosis compared with sham operated mice. And we investigated the effects of rapamycin plus atorvastatin on the regulation of inflammatory cytokines and dyslipidemia to prevent the development of atherosclerosis in CKD-induced apolipoprotein E-deficient (ApoE-/-) mice.

Chapter 2. Materials and Methods

2.1 Experimentally Induced CKD Mice Model (Two-Step Procedure)

All animal studies were approved by the Institutional Animal Care and Use Committee of Ewha Womans University (IACUC-18-036). All experiments carried out on female ApoE-/- mice at eight weeks of age. The animals were maintained in a specific pathogen-free facility set on a 12-h light-dark cycle and were given free access to chow normal diets with sterilized water.

A two-step procedure was used to induce uremia. Briefly, the right kidney was dissected from the adrenal gland and surrounding fat through a 2-cm flank incision and cauterized by Bovie High-Temperature Battery-Operated Cautery (symmetry surgical, TN, USA) except 2 mm around the hilum. Two weeks later, left total nephrectomy was performed through a left flank incision after clipping ureter and renal artery and vein using surgical clip.(31-33) The sham operation used as a control comprised the decapsulation of both kidneys.

After two weeks of recovery, they were divided into four groups (CKD, atorvastatin, rapamycin, and rapamycin + atorvastatin groups) and fed a Western diet (#D12097B; Research Diets, Inc., NJ, USA) for 10 weeks to induce atherogenesis. As indicated, rapamycin (0.5 mg/kg) or atorvastatin (10 mg/kg) were given by oral gavage for 5 days a week for 10 weeks (Figure 1). Compounds of rapamycin and atorvastatin were supplied by Pfizer through

Compound Transfer Program of Pfizer after submitting the proposal and obtaining the approval.

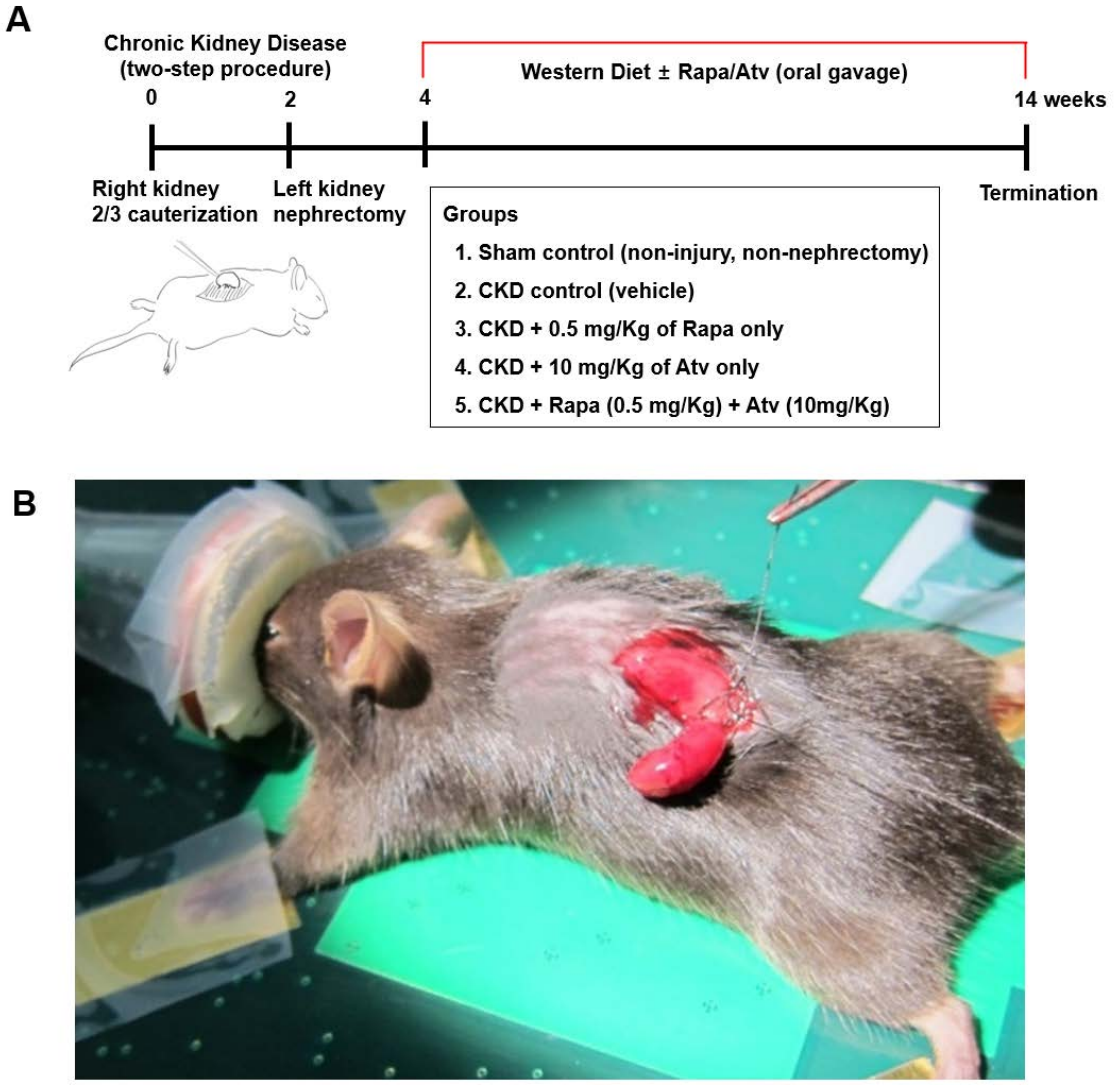

Figure 1. (A) Schematic presentation of the experimental protocol. (B) Two weeks later, left total nephrectomy was performed through a left flank incision. CKD: chronic kidney disease; Rapa: rapamycin; Atv: atorvastatin

2.2 Blood Biochemistry

Whole blood was drawn through the retro-orbital plexus of each deeply

anesthetized mouse using a heparin-treated capillary. Serum was separated by centrifugation at 1,500 g for 15 min and stored at -80 ℃ until analysis. Serum levels of BUN, creatinine, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using a Hitachi 7180 biochemistry autoanalyzer (Hitachi Ltd., Tokyo, Japan). The levels of calcium, phosphate, total protein, and albumin were also measured.

2.3 Atherosclerotic Lesion Analysis

Mice were euthanized with carbon dioxide inhalation, and the hearts and aortas were perfused through the left ventricle with ice-cold phosphate-buffered saline (PBS). Hearts, including the aortic roots, were embedded in frozen-section compound (3801480; Leica, IL, USA) and serially sectioned at 7 ㎛. For en face analysis, the aorta was opened along the longitudinal axis and pinned onto a black wax plate. For measurement of atherosclerotic plaque lesions, the aorta and the heart section were fixed with 10% formalin in PBS and stained using an Oil red O solution. The lesion areas were analyzed using the Axio Vision software (Carl Zeiss, Jena, Germany).

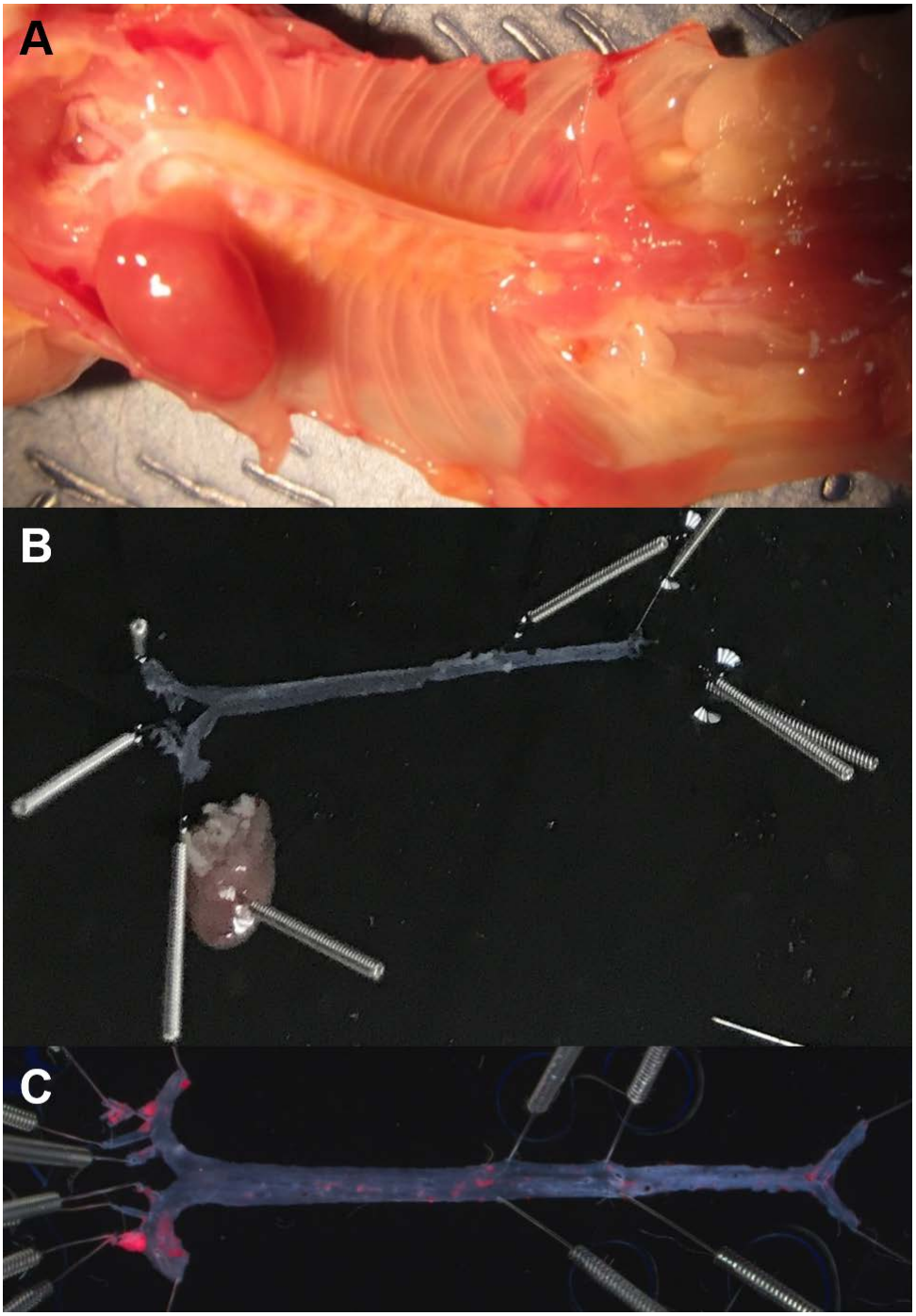

Figure 2. Aorta was perfused through the left ventricle with ice-cold phosphate-buffered saline (A) and was opened along the longitudinal axis and pinned onto a black wax plate (B). Then the aorta and the heart section were fixed with 10% formalin in PBS and stained using an Oil red O solution (C).

2.4 RNA Isolation and Quantitative Real-Time PCR

Total RNA from tissue samples was prepared with the TRIzol reagent (Gibco, CA, USA), and cDNA was synthesized with the Maxime™ RT-PCR PreMix (iNtRON Biotechnology, Korea). Quantitative real-time PCR (SYBR® FAST, Kappa Biosystems, MA, USA) was conducted to determine the relative levels of mRNA using a 7700 Sequence Detector System (Applied Biosystems, CA, USA) and primers for the target mouse genes (Table 1). The data were normalized to the mRNA level of GAPDH in each reaction.

Table 1. Primer Sequences

Gene	Sequence (5' -> 3')	
	Forward primer	Reverse primer
HMGCR	TTTCTAGAGCGAGTGCATTAGCA	GATTGCCATTCCACGAGCTATAT-3'
LXR-a	GATGTTTCTCCTGATTCTGCAAC	AGGACTTGAGGAGGTGAGGAC
ABCG5	CCTGCTGAGGCGAGTAACAA	TGGCACCCACAAGCTGATAG
CYP7A1	CACTCTACACCTTGAGGATGG	GACATATTGTAGCTCCTGATCC
PPAR-γ	AGATTCAGAAGAAGAACCGGAAC	CCGATCTCCACAGCAAATTATAG
ApoA1	GCATGCGCACACACGTAGACTCTCT	CGTCTCCAGCATGGGCATCAGACTA
TNF-a	TGGCCCAGACCCTCACACTCAG	ACCCATCGGCTGGCACCACT
IL-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
IL-1β	GGAGAACCAAGCAACACAAAATA	TGGGGAACTCTGCAGACTCAAAC
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-4	GAATGTACCAGGAGCCATATC	CTCAGTACTACGATGAATCCA
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

2.5 Western Blot Analysis

Total protein was extracted from tissue samples using EzRIPA Lysis buffer containing protease and phosphatase inhibitor cocktail (ATTO, Tokyo, Japan). For analysis of target protein expression, proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated in primary antibodies at 4 ℃ for overnight, and with secondary antibodies for 2 hours at room temperature. Immunoreactive band were visualized and quantified. Antibodies for target protein were purchased as followed: anti-LXRα (Abcam, Cambridge, UK); anti-CYP7A1, anti-PPARγ, anti-IL6 and anti-VCAM1 (Santa Cruz Biotechnology, CA, USA); anti-ABCG1 (Novus Biologicals, CO, USA); anti-ApoA1 (Biodesign, ME, USA); anti-GAPDH and HRP-conjugated goat anti-mouse/rabbit IgG antibodies (GeneTex, TX, USA).

2.6 Fast Protein Liquid Chromatography (FPLC) Analysis

Gel filtration on an FPLC system was used to analyze plasma lipoproteins. Pooled plasma samples were run on two Superose 6 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden) at a flow rate of 0.5 mL/min, with 120 mL of plasma per sample. Cholesterol levels were measured using an enzymatic assay (Wako Pure Chemical Industries Ltd., Osaka, Japan).

2.7 Immunostaining

Monocytes and macrophages were detected with a rat anti-mouse MOMA-2 antibody (ab33451, Abcam, United States). A rabbit anti-mouse α-SMA antibody (C6198, Sigma-Aldrich, Germany) was used for staining smooth muscle cells (SMCs). appropriate fluorescence-labeled secondary antibodies were used according to the manufacturers' protocols. Images were examined using a multicolor digital camera on an IX-81 laser confocal microscope (Olympus, Japan).

2.8 Statistical Analysis

Continuous variables are expressed as the mean ± standard deviation and were compared using the Mann-Whiney U test. Categorical variables were tested with Fisher's exact tests and are expressed as counts and percentages. Body weights were analyzed using a repeated-measures ANOVA followed by Bonferroni's post-hoc test. Analyses were performed with the SPSS Statistical Analytics software (IBM Analytics, NY, USA).

Chapter 3. Results

3.1 Rapamycin Ameliorates CKD-Associated Atherosclerosis

To establish a consistent CKD mouse model, we used a two-step surgical nephrectomy in 8-week-old female ApoE−/− mice. We first evaluated the serum chemistry (Figure 3, Table 2) and found that the levels of blood urea nitrogen (BUN), creatinine, and calcium were markedly increased in the sera of mice subjected to surgical nephrectomy compared to sera in sham-operated mice. However, rapamycin and atorvastatin did not decrease the uremia and the hypercalcemia associated with CKD in mice. No significant difference between groups was seen in the serum phosphate level. CKD mice treated with rapamycin plus atorvastatin gained weight.

To find the effect of rapamycin and atorvastatin on atherosclerotic lesions accelerated by CKD, we established the CKD model as described above, and the mice were further fed a Western diet to induce atherosclerosis. This model was used in all subsequent experiments. Oil red O staining showed that the CKD group exhibited more atherosclerotic plaque formation in the whole aorta than the Sham group (Figure 5A). The rapamycin and rapamycin + atorvastatin groups exhibited significant reductions of the atherosclerotic lesions, whereas the atorvastatin group did not differ from the CKD group. There was no significant difference between the rapamycin and rapamycin + atorvastatin groups in this parameter. We observed similar results when we analyzed the

stained aortic sinuses of mouse hearts (Figure 5B). Together, these findings indicate that rapamycin reduces the atherosclerosis associated with CKD in this model, but atorvastatin has no additional effect on this parameter, alone or in combination with rapamycin.

The content of macrophages in the plaques at the aortic root by immunostaining was similar among groups (Figure 6).

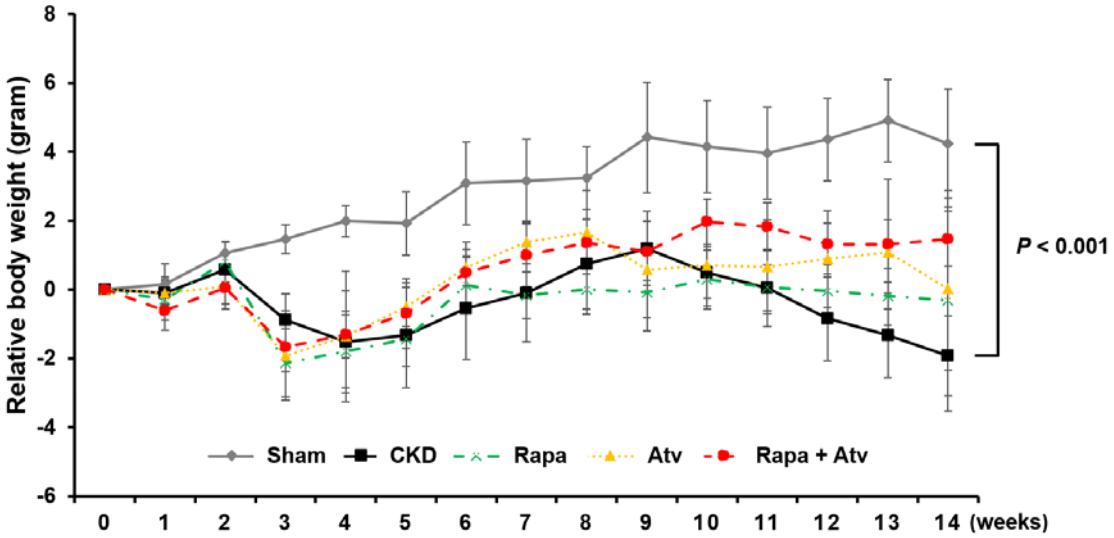

Figure 3. Body weight and levels of blood urea nitrogen and creatinine

Table 2. Body Weight and Laboratory Data

	Sham	CKD	Rapamycin	Atorvastatin	Rapamycin + Atorvastatin
Body weight, g	22.9 ± 1.20	*20.4 ± 2.40	*20.3 ± 3.00	*20.1 ± 4.10	#22.7 ± 1.50
BUN, mg/dL	18.7 ± 3.90	*92.8 ± 14.4	*78.9 ± 32.8	*79.5 ± 19.5	*65.8 ± 25.2
Creatinine, mg/dL	0.28 ± 0.02	*0.47 ± 0.03	*0.65 ± 0.13	*0.59 ± 0.19	*0.56 ± 0.14
B/C ratio	44.4 ± 39.8	*195.3 ± 19.2	*111.8 ± 28.9	*128.8 ± 24.3	*151.3 ± 23.3
Calcium, mg/dL	9.78 ± 0.39	*11.27 ± 1.48	*10.10 ± 0.67	*10.17 ± 0.70	*11.08 ± 0.42
Phosphate, mg/dL	7.23 ± 0.79	8.0 ± 2.0	6.57 ± 1.78	6.73 ± 1.08	8.72 ± 1.00
Total protein, g/dL	6.13 ± 1.40	8.27 ± 3.33	7.94 ± 2.86	4.45 ± 0.31	9.21 ± 4.76
Albumin, g/dL	1.67 ± 0.21	1.77 ± 0.40	1.81 ± 0.39	1.28 ± 0.05	1.99 ± 0.62
Glucose, mg/dL	229.0 ± 26.2	165.7 ± 23.3	191.2 ± 33.4	159.8 ± 24.3	213.2 ± 64.1

BUN, blood urea nitrogen; B/C, BUN/Creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

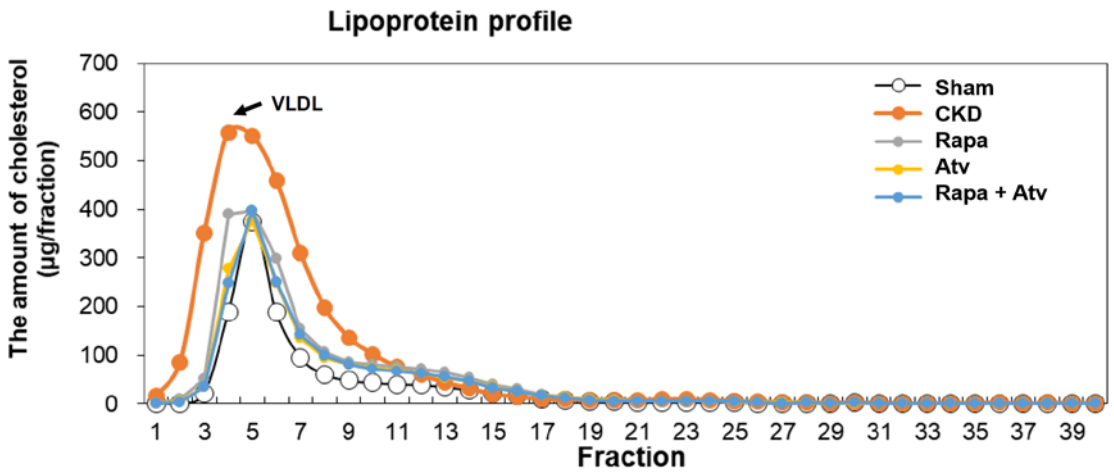

Figure 4. Fast Protein Liquid Chromatography (FPLC) fraction cholesterol levels from pooled plasma samples. The CKD group showed a marked increase in VLDL.

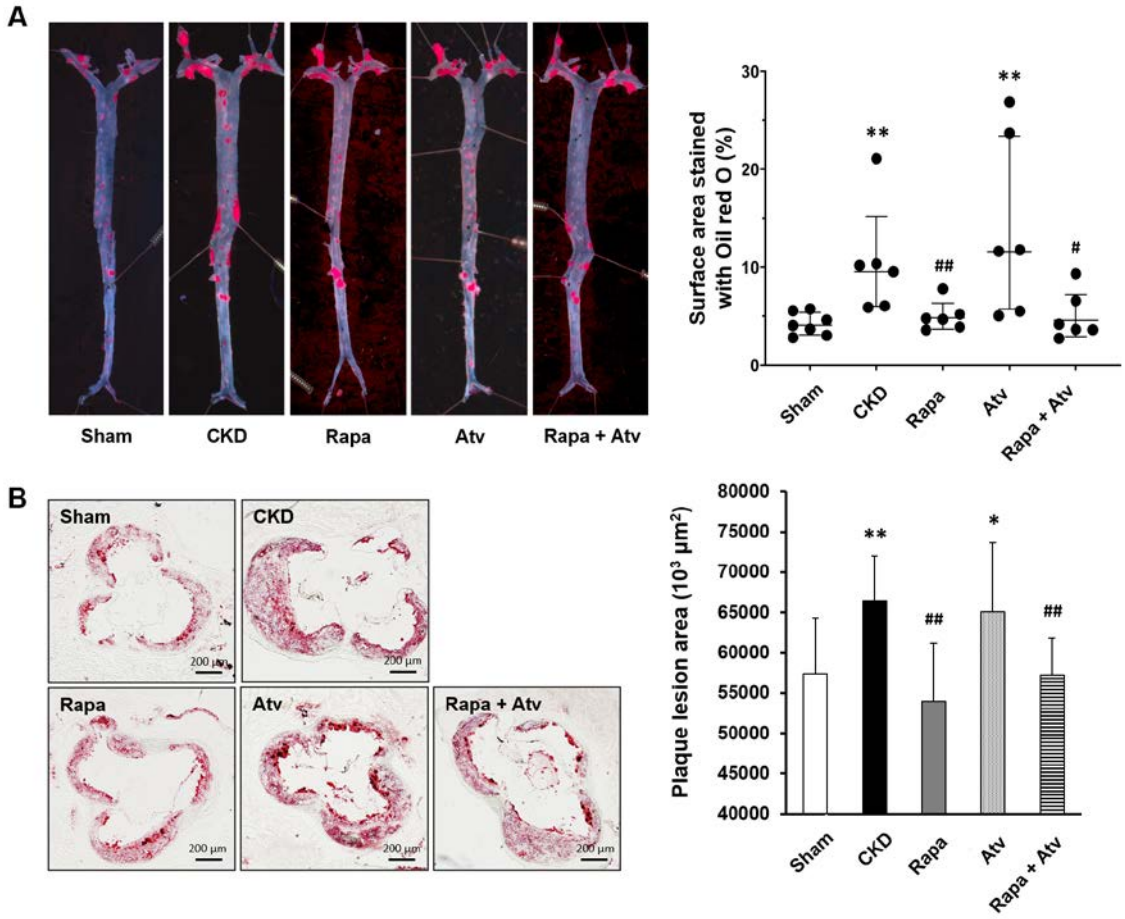

Figure 5. Rapa ameliorates the formation of atherosclerotic plaques, as assessed by Oil red O staining. (A) Representative en face images of whole aortas (left) and quantification of lesion areas from the indicated groups (right). (B) Representative images of frozen sections of aortic sinuses (left) and quantification of plaque areas on aortic sinuses of the indicated groups (right). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

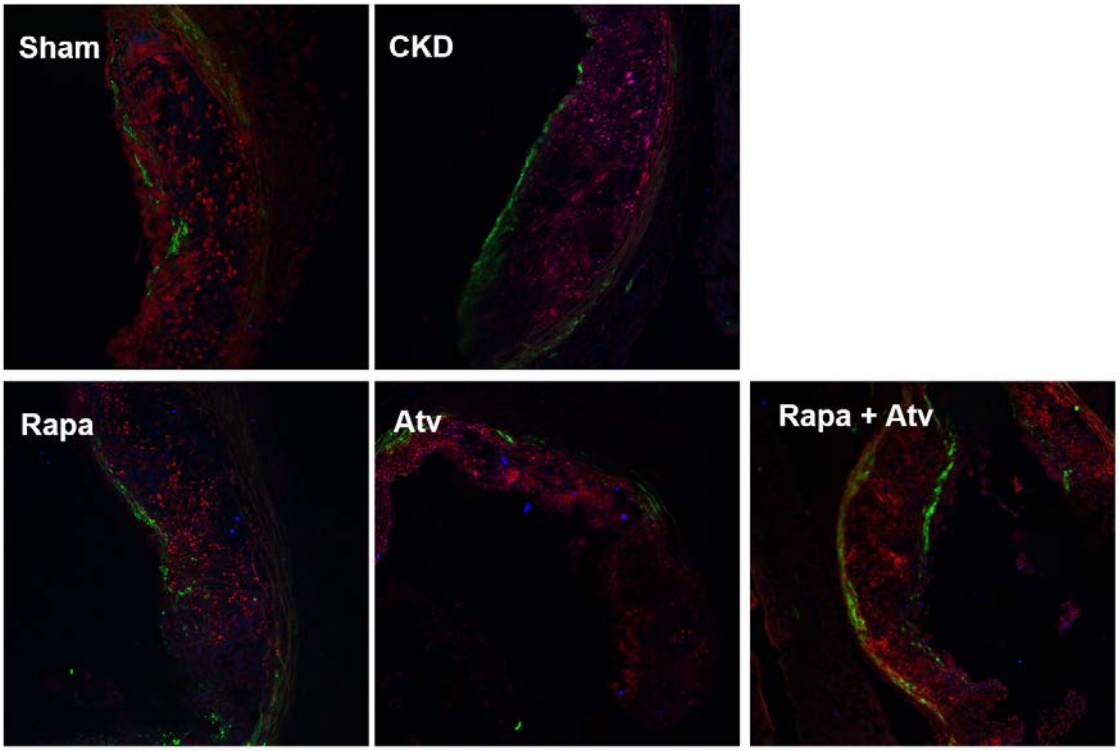

Figure 6. Sections of the aortic sinus were stained with the macrophage marker, Moma-2 (red), to determine macrophage content per plaque size. SM α-actin was used to stain smooth muscle cells (green).

3.2 Combination of Rapamycin and Atorvastatin Improves Lipid Metabolism in CKD Mice

To examine the serum lipid levels in our experimental system, we fasted animals for 4 hours, sacrificed them, and collected whole blood for serum chemistry. The serum levels of total cholesterol and triglyceride were not different between the groups (Figure 7A, B). Circulating LDL-C was significantly more increased in the CKD group than in the Sham and was inhibited in the atorvastatin group. However, neither rapamycin nor rapamycin + atorvastatin affected the LDL-C level elevated by CKD in those groups. The

serum level of HDL-C in the CKD and rapamycin group was similar to that in the Sham group, but the level was markedly elevated in the atorvastatin and rapamycin + atorvastatin groups (Figure 7C, D). Surprisingly, the HDL-C levels in the co-administration of rapamycin and atorvastatin were higher than that seen in the atorvastatin group, with an increase that was twice those seen in the Sham and CKD groups.

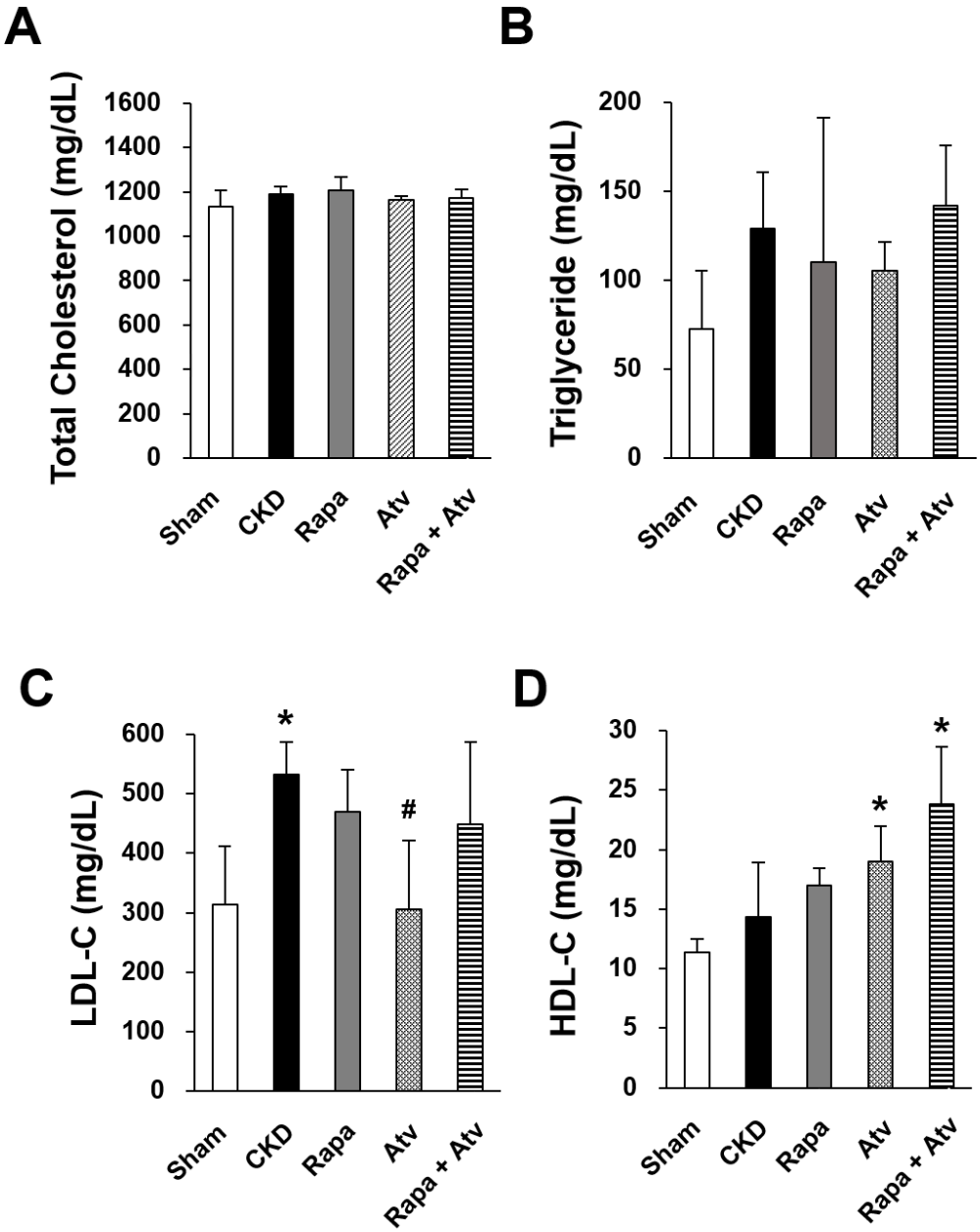

Figure 7. Combined treatment with Rapa plus Atv has beneficial effects on lipid metabolism. Serum lipid profiles for (A) total cholesterol, (B) triglycerides, (D) high-density lipoprotein cholesterol (HDL-C), and (C) low-density lipoprotein cholesterol (LDL-C) from the indicated groups (n = 6-9). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

To explore the effect of rapamycin and atorvastatin on lipid metabolism, we used qRT-PCR to evaluate the mRNA expression levels of genes related to cholesterol metabolism, including HMGCR, LXRα, ABCG5, CYP7A1, PPARɣ, and ApoA1, in the livers of our experimental and control mice (Figure 8).

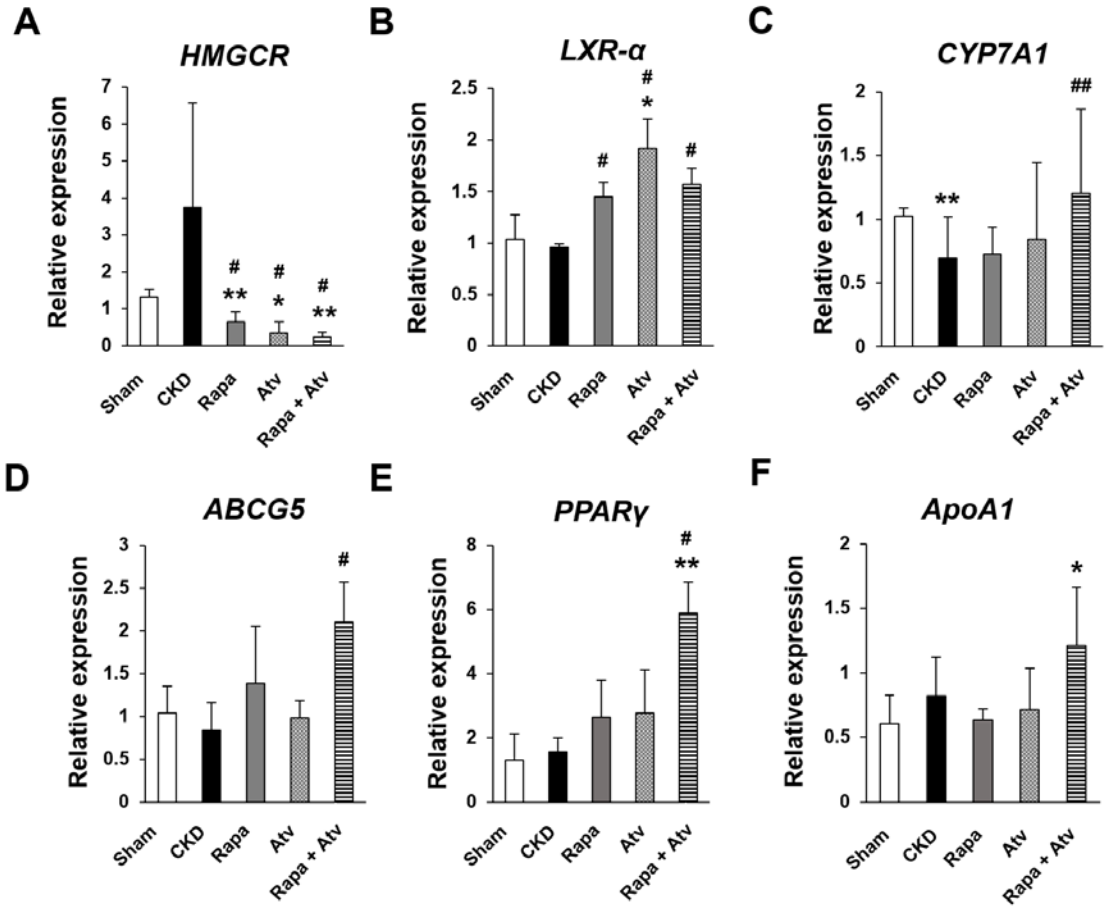

Figure 8. mRNA expression levels of (A) cholesterol metabolism-related genes, (B, C) cholesterol transport-related genes, (D) bile acid biosynthesis genes, and (E, F) lipid and glucose metabolism-related genes in the livers of the indicated groups (n=5-7 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

As expected, CKD increased the mRNA expression level of HMGCR, and this change was inhibited by treatment with rapamycin and atorvastatin. The

HMGCR expression level did not differ between the rapamycin, atorvastatin, and rapamycin + atorvastatin groups, and the levels of all three groups were lower than that of the Sham group. CKD tended to decrease the gene expression levels of LXRα compared to those in the Sham group. The gene expression of LXRα was significantly higher in the rapamycin and atorvastatin groups than in the CKD and Sham groups. The classical pathway of bile acid initiated by CYP7A1 was more inhibited in the CKD group than in the Sham group. The rapamycin + atorvastatin group had significantly higher CYP7A1 expression than did the CKD group. In addition, combined administration of rapamycin and atorvastatin significantly increased the mRNA level of ABCG5, PPARγ and ApoA1 whereas this parameter did not differ between the rapamycin, atorvastatin, Sham, and CKD groups.

We subsequently examined the expression level of proteins related to reverse cholesterol transport, including LXRα, CYP7A1, ABCG1, PPARγ, and ApoA1, in the mouse liver (Figure 9).

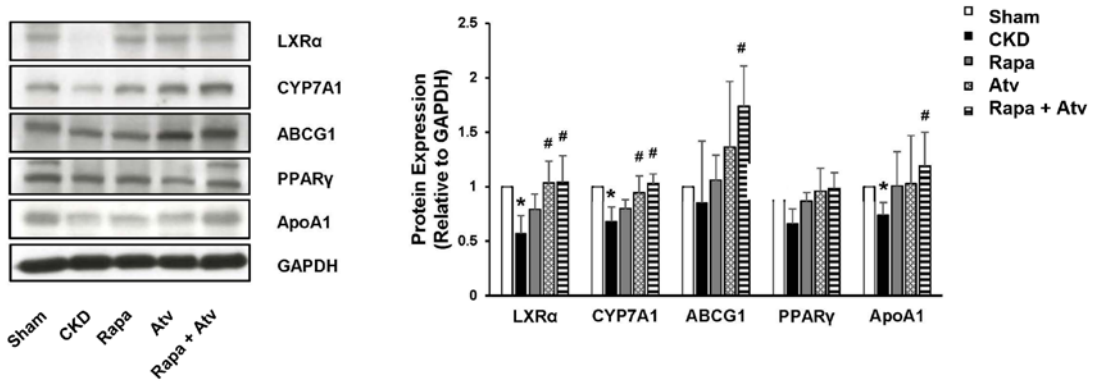

Figure 9. The expression levels of reverse cholesterol transport-related proteins in the livers of the indicated groups (n = 5 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

The CKD group significantly reduced the expression of LXRα, CYP7A1 and ApoA1. The decreased level of LXRα and CYP7A1 were recovered in similar with the Sham group by atorvastatin administration, but the levels were not affected by rapamycin treatment. Interestingly, the rapamycin + atorvastatin group had significantly elevated expression level of LXRα, CYP7A1, ABCG1 and ApoA1 in the liver. The expression level of PPARɣ was slightly lowered in the CKD group, but there was no significant difference between any of the groups. Given the results, we found that co-administration of rapamycin and atorvastatin is more effective in stimulating reverse cholesterol transport and bile secretion than is atorvastatin treatment alone. These data suggest that combining rapamycin with atorvastatin may help to mitigate dyslipidemia in CKD.

3.3 ApoE−/− Mice with CKD Exhibit Up-Regulation of Pro-Inflammatory Cytokine Genes in the Spleen and Aorta, and these Levels Are Reduced by Rapamycin

Next, we used qRT-PCR to investigate the effects of rapamycin and atorvastatin on the expression levels of atherogenesis-related inflammatory cytokines. In the spleen, the mRNA expression of inflammatory cytokines is known to promote atherosclerosis, including TNF-α, IL-6, and IL-1β, which were specifically more increased in the CKD group than in the Sham group (Figure 10A-C). The CKD-related up-regulations of TNF-α and IL-1β were suppressed in the atorvastatin, rapamycin, and rapamycin + atorvastatin groups, and their levels did not significantly differ between these groups. The administration of rapamycin significantly inhibited the CKD-related increase of IL-6 expression, whereas this level was similar among the atorvastatin, atorvastatin + rapamycin, and CKD groups. Interestingly, IL-10, an anti-inflammatory cytokine, was more increased in the CKD group than in the Sham group, and this increase was suppressed in the atorvastatin and rapamycin + atorvastatin groups (Figure 10D).

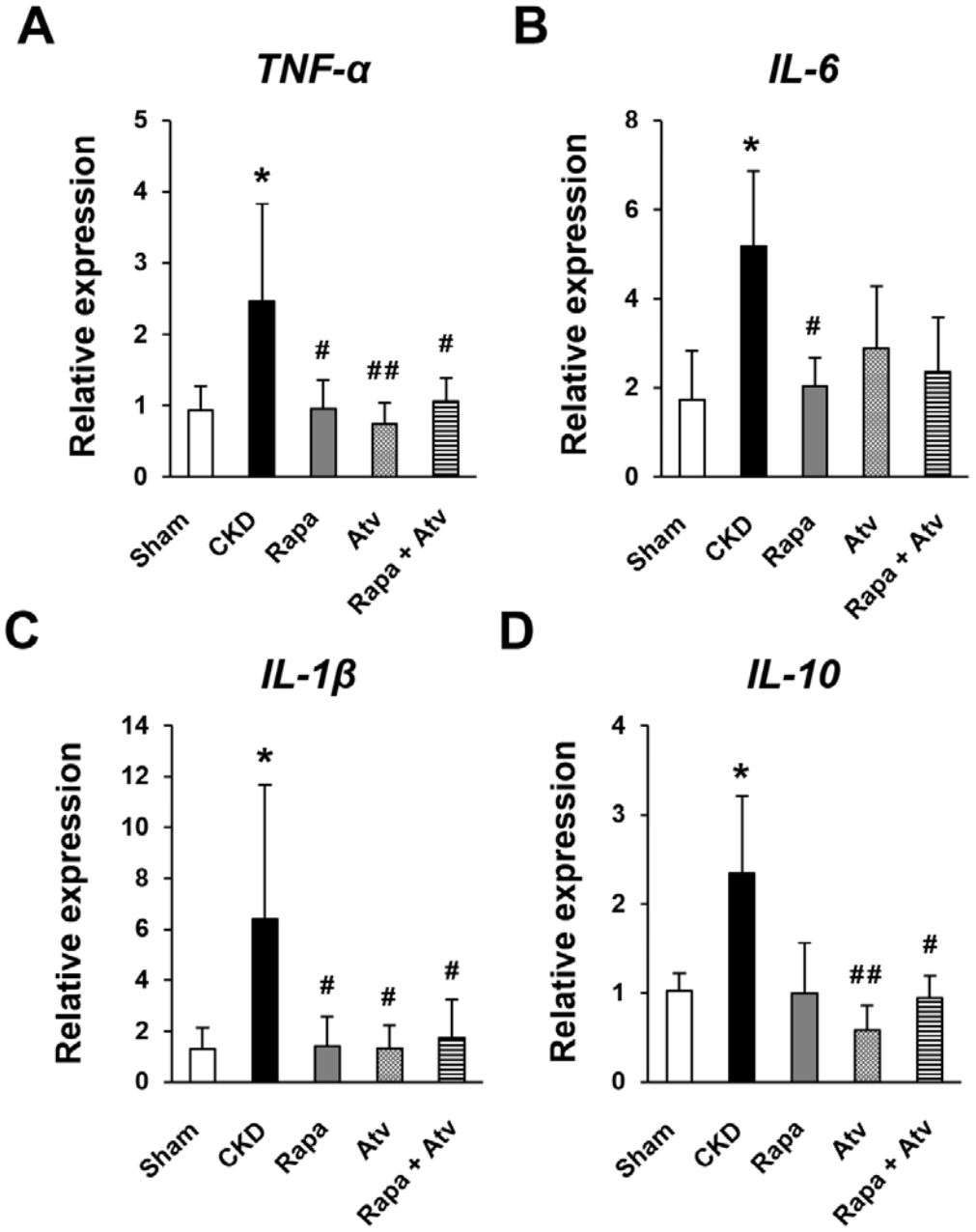

Figure 10. The effects of RAPA and ATV on the CKD-related stimulation of pro atherosclerosis cytokines in ApoE−/− mice. Gene expression levels of inflammatory cytokines in the spleen (n = 5-7 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

In the aorta, the mRNA levels of TNF-α and IL-4 were higher in the CKD group than in the Sham group (Figure 11A, 11B, whereas the rapamycin group had significantly lower levels of TNF-α and IL-4 than did the CKD group. However, atorvastatin and rapamycin + atorvastatin did not decrease their levels like rapamycin. Surprisingly, the mRNA expression level of IL-6 did not differ in any of the groups (Figure 11C). The mRNA level of IL-10 was significantly increased in the rapamycin group over that in the Sham and CKD groups (Figure 11D), but this significant increase was not seen in the rapamycin + atorvastatin group.

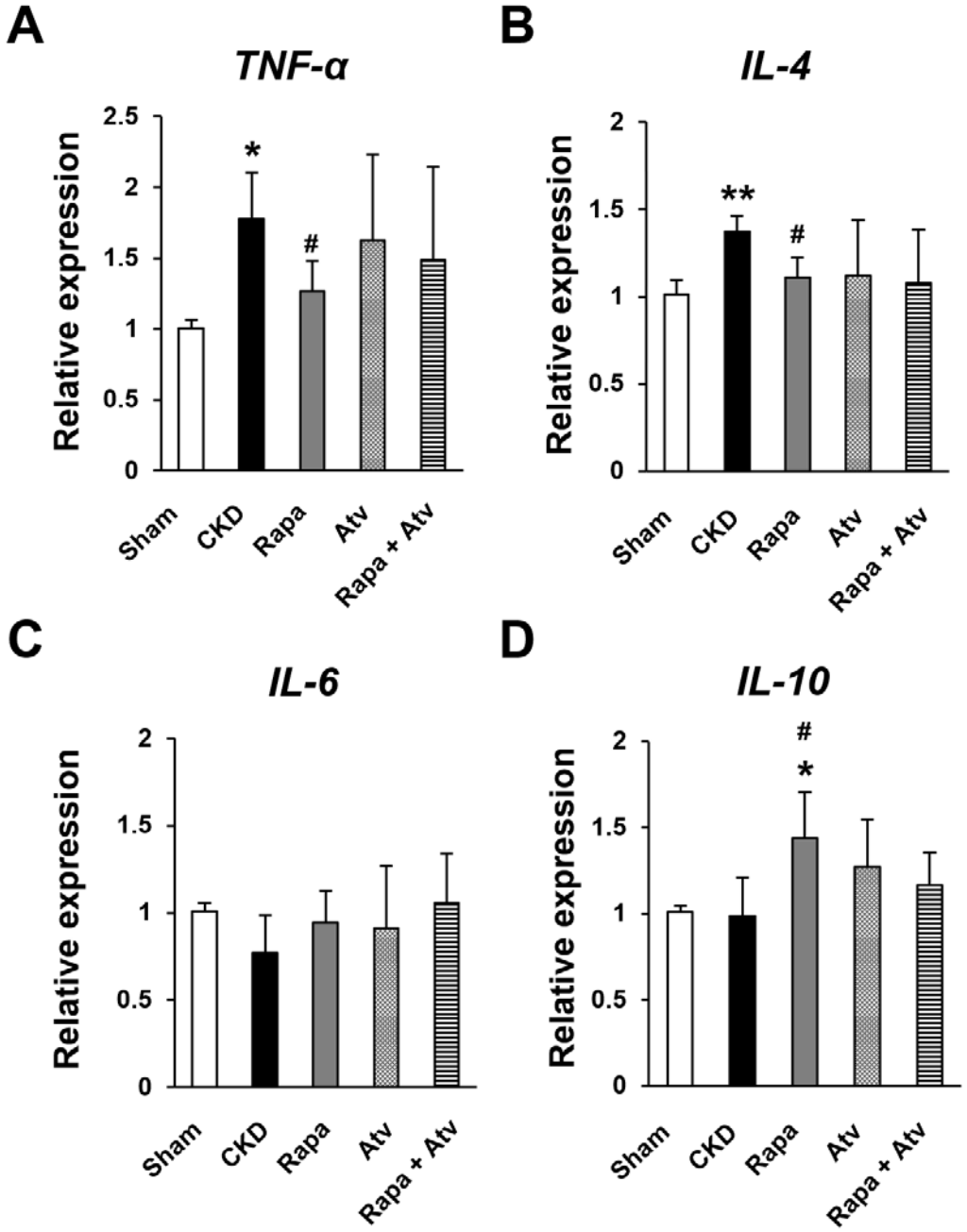

Figure 11. The effects of Rapa and Atv on the CKD-related stimulation of pro atherosclerosis cytokines in ApoE−/− mice. Gene expression levels of inflammatory cytokines in the aorta (n = 5-7 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

These results suggest that the administration of rapamycin helps decrease the levels of atherosclerosis-promoting cytokines and increases those of atherosclerosis-suppressing cytokines, but that combined treatment with rapamycin and atorvastatin does not appear to show synergistic effects.

Chapter 4. Discussion

We herein provide the first report on how combined treatment with rapamycin and atorvastatin affects the atherogenesis stimulated by CKD. In our study, the oral administration of rapamycin in CKD-induced ApoE−/− mice ameliorated atherosclerotic lesions and inhibited the mRNA expression levels of pro-inflammatory cytokines in the spleen and aorta. These data confirm that rapamycin plays an athero-protective role by reducing the pro-inflammatory burden in CKD. Although atorvastatin did not additionally reduce the aortic lesions, the combined use of atorvastatin markedly increased the serum levels of HDL-C in ApoE−/− mice with CDK. Thus, our results suggest that the combination therapy of rapamycin plus atorvastatin has a beneficial effect in alleviating the atheroprone environment of CKD.

4.1 Experimentally Induced CRF Mice Model (Two-Step Procedure)

The formation of atherosclerotic lesions can vary by the stage of renal failure. An electronic cautery-based renal injury mouse model has been used in various CKD studies because such mice exhibit substantial increases in their serum levels of BUN and creatinine.(32, 33) However, these methods did not show sufficient or consistent renal impairment in our preliminary experiments. We instead used high-temperature battery-operated cautery to forcefully damage the kidney and found that this yielded a consistent CKD model. The serum

calcium and phosphate levels of operated mice were significantly elevated, supporting the idea that our model is suitable for the mechanistic study of CKD.(33)

4.2 Combination of Rapamycin and Atorvastatin Improves Lipid Metabolism

rapamycin has been shown to significantly suppress atherosclerosis in studies using animal models with normal renal function.(24, 34) But there are concerns about adverse effects that may be associated with rapamycin treatment, such as dyslipidemia. Several researchers suggested that combined treatment with a statin would reduce the possibility of rapamycin-induced dyslipidemia, resulting in additional amelioration of atherosclerosis.(34) This prompted us to examine the potential of atorvastatin to counter this disadvantage of rapamycin treatment. Consistent with previous findings, our results showed that atorvastatin lowered the mRNA expression of HMGCR, and that rapamycin also inhibited the expression of this mRNA to a similar level.(35) This suggests that the combination of atorvastatin with rapamycin may not show a synergic lipid-lowering effect, considering that they both target HMGCR. However, our study demonstrated that CKD mice treated with rapamycin plus atorvastatin gained weight (Figure 3) and exhibited stabilization of the HDL-C level (Figure 5). Furthermore, the combination therapy increased the levels of ABCG5 and cholesterol transport-related genes, and increased bile acid biosynthesis

through the activation of CYP7A1. It also promoted lipid metabolism through PPARɣ and ApoA1. These results suggest that the combined treatment could improve general conditions and long-term mortality in mice with renal failure, and thus support the use of statins in CKD.

4.3 Rapamycin And Atorvastatin Markedly Decreased the Gene Expression Levels of Atherosclerosis-Promoting Inflammatory

Dysregulation of cytokines in CKD is associated with a significant decrease in cytokine secretion, given that the kidney is a main organ for eliminating cytokines.(11) rapamycin was previously shown to target pro-inflammatory cytokines and mTOR activation induced by chronic consumption of a high-fat diet.(36-38) Indeed, we found that rapamycin, atorvastatin, and rapamycin + atorvastatin all markedly decreased the gene expression levels of atherosclerosis-promoting inflammatory cytokines such as TNF-α, IL-6and IL-1β in the spleen. Interestingly, the mRNA level of the anti-inflammatory cytokine, IL-10, was increased in the spleen tissues of the CKD group. This is considered to be a compensatory mechanism intended to correct inflammation in CKD.(39) In the aorta, rapamycin specifically decreased the level of lL-4 and increased the level of IL-10, indicating that it exerts anti-inflammatory effects to prevent atherogenesis. Conversely, these levels were similar in the atorvastatin, rapamycin + atorvastatin, and CKD groups. Several reports have suggested that atorvastatin can have inhibitory effects on atherosclerosis in

non-CKD models.(40, 41) Thus, the previous and present results demonstrate that rapamycin and atorvastatin can both regulate the systemic inflammation environment in CKD patients, but that these effects are not synergic.

4.4 Atorvastatin in CKD

Statin medications have been shown to have numerous health benefits, particularly in reducing the risk of cardiovascular events. However, high statins may be necessary for patients with renal failure to achieve these benefits.(42) There are several different types of statins available, including simvastatin, lovastatin, rosuvastatin, and atorvastatin.(43) These medications can have different beneficial effects and side effects.(44-46)

Atorvastatin and rosuvastatin are commonly prescribed in patients with renal failure.(47) Studies have shown that atorvastatin may have some advantages over rosuvastatin in terms of preserving the glomerular filtration rate and no dose adjustment in end-stage renal failure.(48) Therefore, atorvastatin may be considered the preferred statin medication for patients with renal impairment.(49, 50)

4.5 Conclusion

In conclusion, we herein show that rapamycin can play a critical role in reducing the development of atherosclerosis in the aortas of CKD-induced ApoE−/−

mice through alleviating systemic inflammation. Although the co-administration of atorvastatin did not further reduce atherosclerosis in the aorta, it improved the lipid profile and bile acid metabolism in these mice. This first animal experiment with rapamycin and atorvastatin demonstrated the potential for combining these two medications to provide compensatory effects in alleviating cardiovascular risk in individuals with CKD, beyond the individual effects of each agent.

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국문 초록

배경 및 목적: 만성신부전은 전신적인 pro-inflammatory 상태로 죽경화증의 발생이 증가하여 주요 심혈관계 질환이 증가한다. 라파마이신(rapamycin)은 죽경화증의 억제 작용을 하는 것으로 알려져 있으나 이상지혈증을 유발할 수 있다. 따라서 아토바스타틴(atorvastatin)과 같은 지질조절제(lipid-lowering agents)의 병용 투여를 권장한다. 그럼에도 불구하고 만성신부전에서 라파마이신과 아토바스타틴의 병용투여의 효과에 대한 연구는 없다. 본 연구에서는 신부전이 유도된 아포지단백질 E결핍(apolipoprotein E-deficient, ApoE-/-)에서 라파마이신과 아토바스타틴의 병용투여가 죽경화증 발생과 혈중 지질대사에 미치는 영향을 알아보고자 한다.

방법: 마우스를 5개 군으로 무작위로 나눈 후 1개 군에서 헛수술(sham-operation)과 4개 군에서 5/6에 해당하는 신장절제를 시행하여 신부전을 유도했다(신부전군, 아토바스타틴군, 라파마이신군, 라파마이신+라파마이신군). 아토바스타틴(10 mg/kg)이나 라파마이신(0.5 mg/kg)을 해당 군에 10주 동안 매일 경구 위영양(oral gavage)했다.

죽경화증 병변의 확인을 위해 대동맥과 대동맥굴(aortic sinus)를 Oil Red O염색했다. 간에서 qRT-PCR과 Western blot을 이용하여 지질대사 유전자와 단백질의 발현을 확인했다. 대동맥과 비장에서 pro/anti-inflammatory cytokine의 유전자의 발현정도를 측정했다.

결과: 라파마이신군과 라파마이신+아토바스타틴군의 대동맥과 대동맥굴의 Oil Red O염색에서 신부전군과 비교하여 의미 있는 죽경화증 발생의 감소가 관찰되었다. 하지만 아토바스타틴군에서 죽경화증 발생 감소는 없었다. 라파마이신과 아토바스타

틴의 병용 투여는 혈청의 지질 프로필을 향상하였고 간에서 콜레스테롤 수송 (ABCG5), 담즙합성(CYP7A1) 그리고 지질대사(PPARɣ, ApoA1)와 연관된 유전체의 발현이 증가했다.

신부전군은 비장에서 TNF-α, IL-6and IL-1β과 대동맥에서 TNF-α and IL-4의 atherosclerosis-promoting cytokines의 유전체의 발현이 증가했다. 라파마이신의 투여는 이들 유전자의 감소와 연관이 있었다. 아토바스타틴군과 라파마이신+아토바스타틴군은 비장에서 TNF-α와 IL-1β를 감소시켰다.

결론: 본 연구는 신부전 유도 ApoE-/-마우스에서 라파마이신은 inflammatory cytokine의 발현의 조절을 통해 의미 있게 죽경화증의 발생을 감소시켰고 아토바스타틴의 병용 투여는 추가적인 죽경화증의 발생을 억제하지는 않았으나 지질 대사를 향상시켰다.

주요어: 만성신부전, 죽경화증, 라파마이신, 아토바스타틴, 병용투여, 사이토카인

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