

Aliskiren Inhibits Neointimal Matrix Metalloproteinases in Experimental Atherosclerosis

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Background: The renin-angiotensin system (RAS) plays an important role in atherosclerosis. Acting via the angiotensin II receptor, type 1, oxidative stress increases and contributes to endothelial dysfunction and vascular inflammation. Renin exerts effects through a renin receptor causing an increase in the efficiency of angiotensinogen cleavage and facilitates angiotensin II (Ang II) generation and action on cell surfaces. Ang II enhances proliferation and migration of vascular smooth muscle cells, indicating a direct involvement of the RAS in smooth muscle cell proliferation during neointimal formation. Aliskiren, a direct renin inhibitor, is a new oral antihypertensive drug. However, the role of the direct renin inhibitor in neointimal formation and vascular matrix metalloproteinases remains unclear.

Methods: To investigate the effects of aliskiren on the expression of vascular matrix metalloproteinases, we evaluated the aortic neointimal formation of high-cholesterol-fed animals after vascular injury in vivo and the cellular function of the tumor necrosis factor- α stimulated human aortic smooth muscle cells in vitro. Thereafter, we evaluated vascular expression (by western blot), activity (by gelatin zymography) and molecular pathway.

Results: In this study we demonstrated that aliskiren reduced neointimal hyperplasia in hypercholesterolemic rabbits after vascular injury and the expression of matrix metalloproteinases in the neointima. Aliskiren also inhibited the expression and activities of matrix metalloproteinases on tumor necrosis factor- α (TNF- α)-stimulated human aortic smooth muscle cells via the mitogen-activated protein kinase pathway.

Conclusions: The present study showed that aliskiren inhibited the expression of vascular matrix metalloproteinases. With these results, we have better clarified the potential role of renin inhibitors in human atherosclerosis.

Key Words: Matrix metalloproteinases • Neointimal hyperplasia • Renin inhibitor

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease, and many factors contribute to the atherosclerotic pro-

cess.¹ Hypercholesterolemia, hypertension, smoking, diabetes, and genetics are associated with the onset of atherosclerosis, and the renin-angiotensin system (RAS) plays an important role in the process of atherosclerosis.² Angiotensin II (Ang II) is the major contributor to hypercholesterolemia-induced atherosclerosis.^{3,4} Acting via the angiotensin II receptor type 1 (AT1 receptor), Ang II increases oxidative stress to cause endothelial dysfunction and vascular inflammation.

Renin mRNA and protein are found predominantly in the medial smooth muscle cell layer and are present at increased levels after balloon injury.⁵ Neointima formation of the vascular wall is related to the phenotypic flexibility of vascular smooth muscle cells (VSMCs). Neointimal smooth muscle cells (SMCs) are derived from

Received: August 5, 2015 Accepted: October 12, 2015

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medial SMCs migrating to the site of inflammation.⁶ In the neointima, SMCs proliferate and undergo transformation from a contractile to a synthetic phenotype.

Ang II potentiates the proliferation and migration of cultured VSMCs, indicating a direct involvement of the RAS in SMC proliferation during neointima formation.⁷ Renin exerts dual effects through a renin receptor which is distinct from the actions that lead to the production of angiotensin and aldosterone. The renin receptor triggers intracellular signals by activating the extracellular signal-regulated kinases (ERK)1/ERK2 pathway. The renin receptor also acts as a cofactor by increasing the efficiency of angiotensinogen cleavage by receptor-bound renin, thereby facilitating Ang II generation and action on the cell surface.⁸

Aliskiren, an oral renin inhibitor, is a new antihypertensive drug. The blood pressure-lowering effects of aliskiren in patients with mild-to-moderate hypertension are comparable to the angiotensin receptor blockers, irbesartan and losartan.^{9,10} Recent studies have demonstrated that aliskiren augments basal and acetylcholine-stimulated nitric oxide production in Watanabe heritable hyperlipidemic rabbits.¹¹ Aliskiren also reduces atherosclerosis in low-density lipoprotein (LDL) receptor *-/-* mice fed a fat-enriched diet;¹² however, the effects of aliskiren in neointimal formation in atherosclerosis are unknown.

In the current study we used an *in vivo* atherosclerotic rabbit with vascular injury model and human aortic smooth muscle cells (HASMCs) *in vitro* treated with tumor necrosis factor- α (TNF- α) to evaluate the effects of aliskiren in neointimal formation and expression of vascular matrix metalloproteinases (MMPs).

MATERIALS AND METHODS

In vivo study

Animal model

Twenty-four adult male New Zealand White rabbits (2.5-3 kg) were provided 60 mg/kg/day of commercial normal chow diet and water for 2 weeks. The rabbits were then randomly divided into 6 groups, with 6 rabbits in each group. Animals in group 1 were continuously fed with normal chow diet (ND) and served as the control. Animals in group 2 were fed a 2% high-choles-

terol (HC) diet (Purina Mills Inc., MS, USA) for 6 weeks. Animals in group 3 were fed a HC diet +3 mg/kg/day of aliskiren by osmotic mini-pump administration and group 4 were fed a HC diet + 10 mg/kg/day of aliskiren for 6 weeks. At the end of the third week, the abdominal artery was injured with a balloon in the four groups of rabbits. Briefly, a 3F Fogarty embolectomy catheter (Biosensor, CA, USA) was inserted through the femoral artery of anesthetized rabbits and passed to the abdominal aorta (16 cm), inflated with normal saline, and withdrawn four times. Heparin (100 units/kg) was administered immediately after the balloon-injury process. The animals were sacrificed at the end of the third week after balloon injury; the abdominal aortas were cut into 5 segments. A small part of each arterial segment was obtained, immersion-fixed with 4% buffered paraformaldehyde, paraffin-embedded, and cross-sectioned for morphometry and immunohistochemistry.

Morphometric analysis and immunohistochemical staining

Morphometric analyses were performed on hematoxylin-eosin-stained cross-sections for each artery with Image-Pro Plus (Media Cybernetics Inc., Rockville, MD, USA). The areas of intima hyperplasia were quantified by computer-assisted planimetry, and the extent of the lesions was expressed as a proportion of the total surface area (surface area of lesions/total surface area of the thoracic aorta) and a ratio of the intima-to-media. Immunohistochemical staining was performed on serial paraffin-embedded sections (5 μ m thick) from rabbit abdominal aortas using anti-MMP-2 (Calbiochem, San Diego, CA, USA), and anti-MMP-9 antibodies (Calbiochem).

In vitro study

Cell cultures

HASMCs were purchased from Cascade Biologics. HASMC were cultured in Dulbecco's Modified Eagle Medium (Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 5% CO₂ at 37 °C. For all experiments, early passage of HASMCs were grown to 80-90% confluence and made quiescent by serum starvation (0.1% FBS) for at least 24 h.

MTT assay

Cell viability was assessed using the 3-(4,5-Dime-

thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated (5×10^4) in 96-well plates and treated with various concentrations of TNF- α and aliskiren for 24 h. Then, MTT (5 mg/ml) was added for 4 h, then the culture medium was removed and the cells were dissolved in 0.04 N HCl/ isopropyl alcohol. The optical density (OD) at 570 nm and 630 nm was measured using a microplate reader.

Migration assay

Matrigel-coated filter inserts (8 mm pore size) that fit into 24-well invasion chambers were obtained from Becton, Dickinson & Co. (NJ, USA). HASMCs (5×10^4 cells/well) to be tested for invasion were detached from the tissue culture plates, washed, resuspended in conditioned medium collected from HASMCs that were or were not treated with TNF- α or aliskiren + TNF- α for 24 h, then added to the upper compartment of the invasion chamber. Five hundred microliters of the same conditioned medium was added to the lower compartment of the invasion chamber.

Western blot and gelatin zymography analyses

HASMCs were pretreated with aliskiren for 18 h followed by TNF- α stimulation for 24 h. The conditioned media were collected and western blot and gelatin zymography analyses were performed for MMP-2 and -9.

Statistical analyses

Results are shown as the means and standard deviations. The difference in mean values among different groups was analyzed by one-way ANOVA and subsequent *post hoc* Dunnett's test. A $p < 0.05$ was considered statistically significant.

RESULTS

Aliskiren inhibits neointimal hyperplasia in cholesterol-fed endothelium-denuded rabbits

The effect of aliskiren on neointimal hyperplasia was quantified by histomorphometric analysis of the abdominal aortic arterial cross-sections on day 21 after the injury. A significant reduction in the neointimal area and thickness in the injured arteries of rabbits in the low- and high-dose aliskiren groups was noted (Figure 1).

Aliskiren inhibits MMP-2 and -9 expression in the neointima in cholesterol-fed endothelium-denuded rabbits

The hypercholesterolemic group showed marked thickening of the neointima, which exhibited strong expression of MMP-2 and -9. Weaker expression of MMP-2 and -9 in the low- and high-dose aliskiren groups was shown compared to the hypercholesterolemic group (Figure 2).

Cytotoxicity of TNF- α and aliskiren on HASMC

The cytotoxicity of TNF- α and aliskiren for HASMCs were measured using a MTT assay. The different concen-

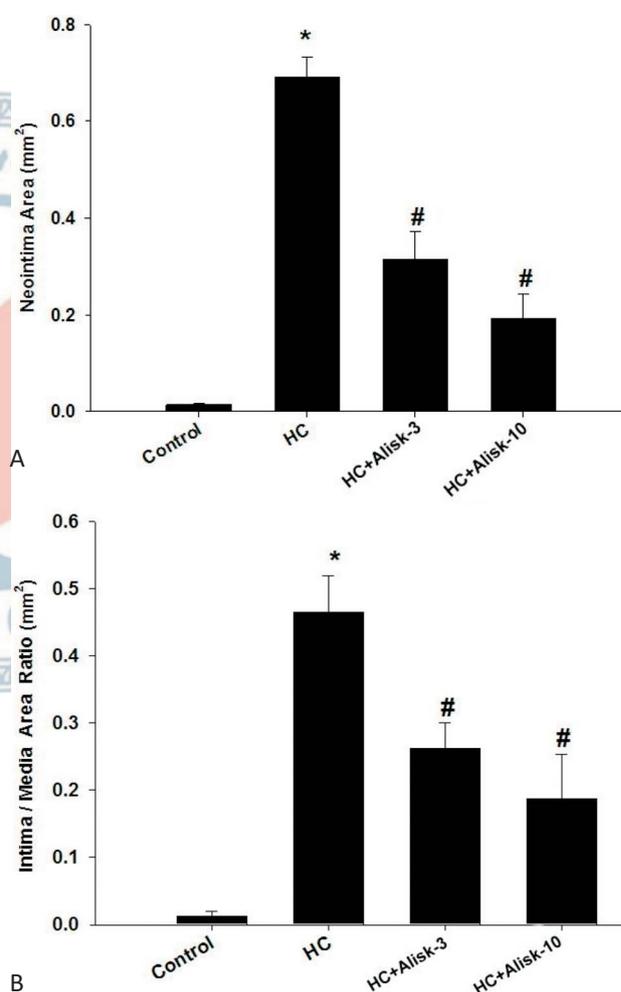


Figure 1. Extent of intimal hyperplasia, expressed as intima/media area ratio and neointimal area of aortas in different groups of rabbits. The ratio was significantly reduced in low- and high-dose aliskiren (Alisk) groups. Data are expressed as the mean \pm standard error of the mean (SEM). * means $p < 0.05$ vs. control group; # means $p < 0.05$ vs. hypercholesterolemic (HC) group; scale bar, 15 μ m.

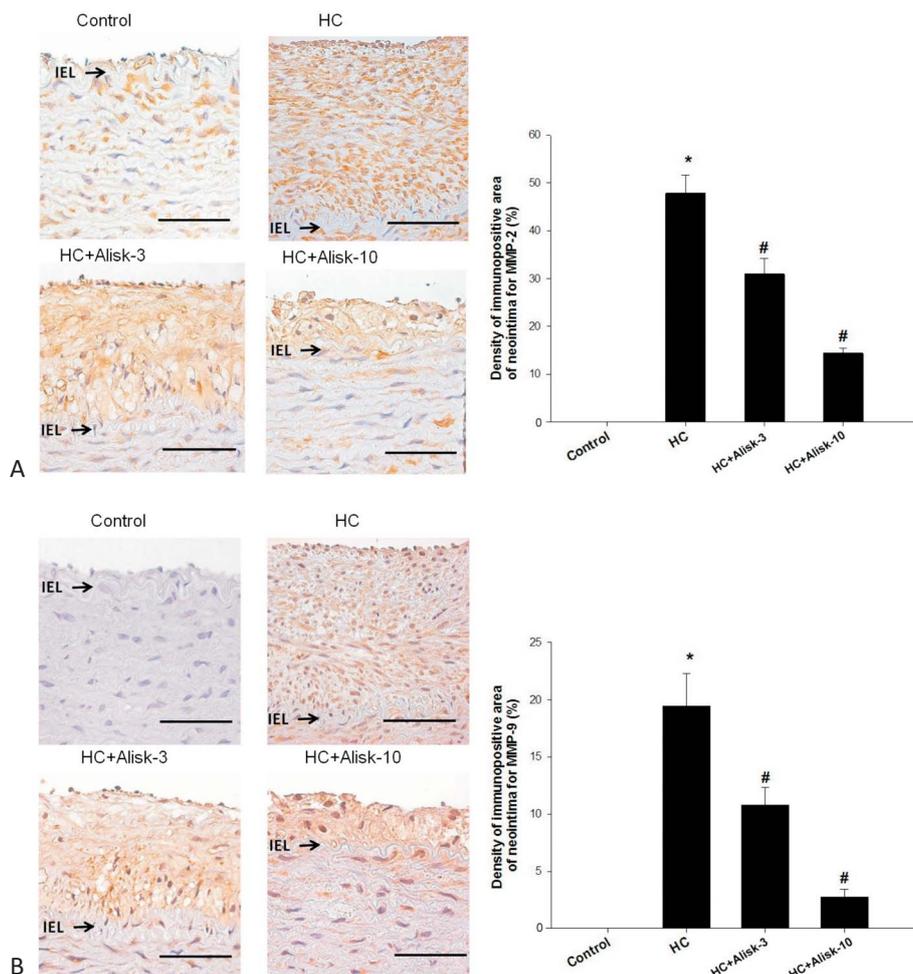


Figure 2. Detection of protein expression in serial sections of abdominal aortas from different groups. (A) The expression of MMP-2. Strong staining was observed in the thickened neointima of the high cholesterol (HC) diet group. Weak staining was observed in parts of the less thickened neointima of the low- and high-dose aliskiren (Alisk) groups. (B) The expression of MMP-9. Strong staining was apparent in thickened neointima of the high cholesterol diet group. Weak staining was observed in parts of the less thickened neointima of the low- and high-dose aliskiren groups. Scale bar, 15 μ m.

trations of TNF- α (Figure 3A) and aliskiren (Figure 3B) were determined. After treatment with TNF- α , the expressions of MMP-2 and -9 were increased, including the total amount of proteins and activities (Figure 3C and 3D). Additionally, the migration of HASMC was increased by TNF- α treatment (Figure 3E).

Aliskiren inhibited the migration of HASMCs and the expression of MMPs on TNF- α -treated HASMCs

Aliskiren significantly inhibited the migration of HASMCs after TNF- α stimulation (Figure 4A). The expression and activities of MMPs on TNF- α -treated HASMCs were also decreased after aliskiren treatment (Figure 4B and 4C).

Effects of aliskiren on the inhibitor of ERK and JNK phosphorylation

Aliskiren significantly suppressed MAPK stimulated by TNF- α stimulated HASMCs. TNF- α stimulated MAPK signaling pathways, including ERK and JNK, in which phosphorylated signals decreased after aliskiren treatment (Figure 5).

DISCUSSION

Aliskiren has the ability to protect against end organ damage. The target organ-protective effects of aliskiren are being investigated in ASPIRE HIGHER, the

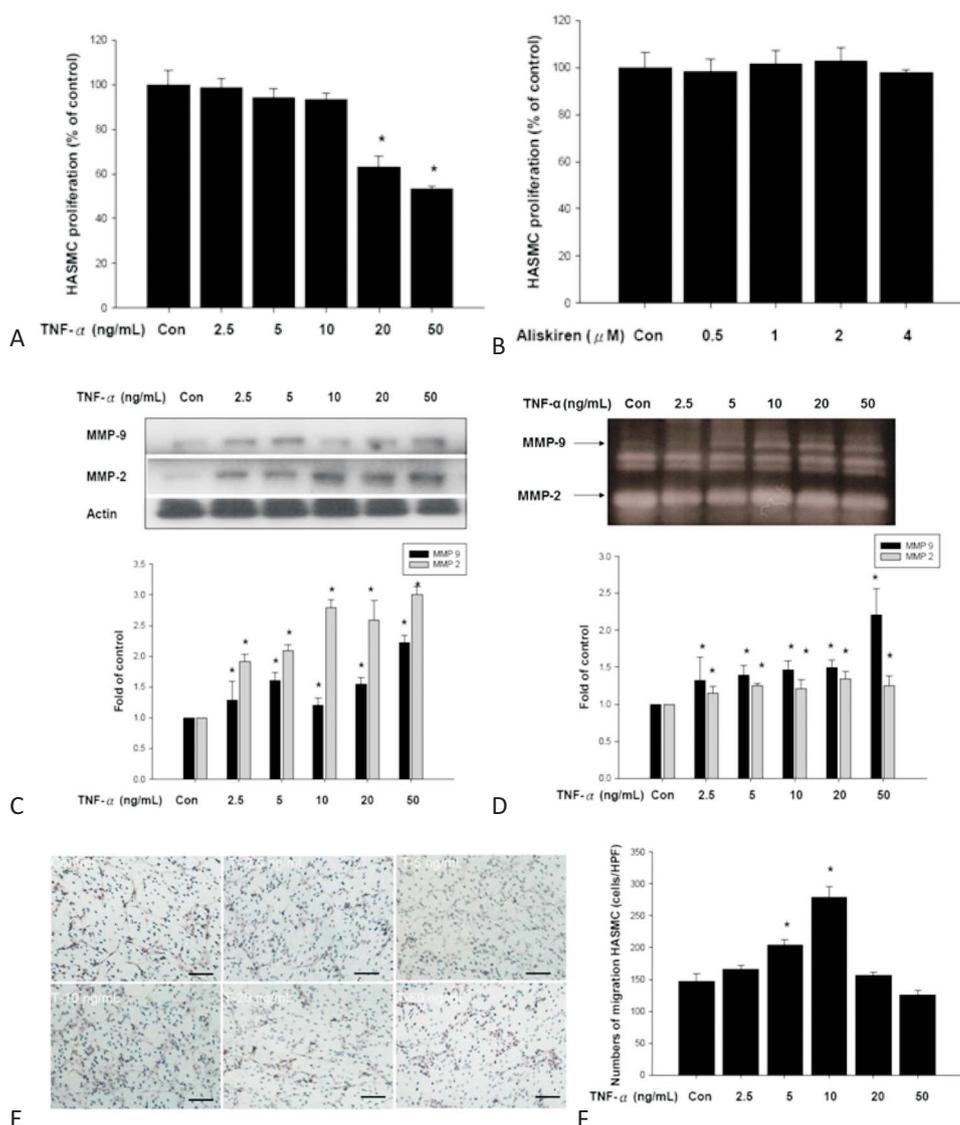


Figure 3. The effects of tumor necrosis factor- α (TNF- α) (A) and aliskiren (Alisk) (B) on human aortic smooth muscle cells (HASMCs) viability. The expression of matrix metalloproteinases (C and D) and migration on TNF- α -induced HASMC (E, F) is shown. The results are expressed as the mean \pm SEM from three triplicate experiments. * means $p < 0.05$, compared with untreated control group; # means $p < 0.05$, compared with TNF- α stimulated group without aliskiren treatment; scale bar, 75 μ m.

largest clinical trial program involving cardio-renal disease.¹³

The Aliskiren in Left Ventricular Hypertrophy (ALLAY) trial compared the effects of aliskiren (300 mg) and losartan (100 mg) alone and in combination on left ventricular hypertrophy based on magnetic resonance imaging assessment in hypertensive patients. It was determined that aliskiren monotherapy was in fact statistically non-inferior to losartan in reducing the left ventricular mass index.¹⁴

The first large study to assess the renoprotective effects of aliskiren was through the investigation Aliskiren in the Evaluation of Proteinuria in Diabetes (AVOID), in which 599 patients with diabetes, nephropathy, and proteinuria were treated with losartan (100 mg) followed by the addition of placebo or aliskiren (300 mg). Adding aliskiren to losartan provided a further 20% reduction in the urinary albumin-to-creatinine ratio compared with the addition of placebo. Nearly twice as many patients in the aliskiren group achieved a reduc-

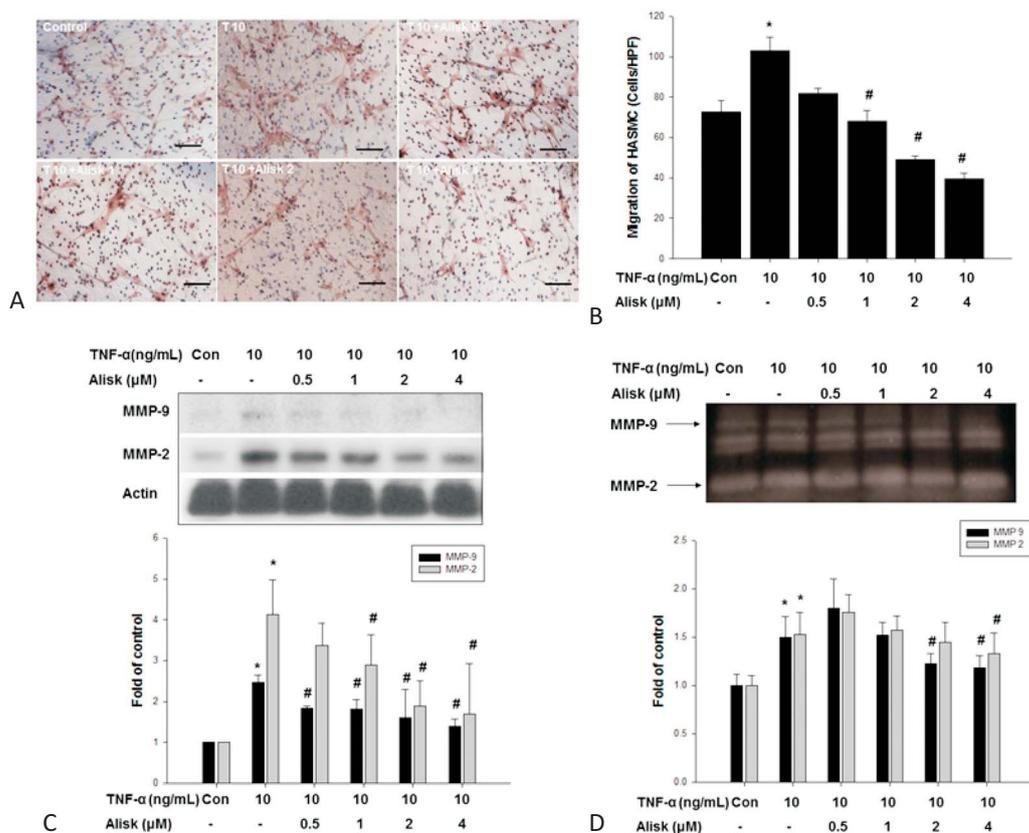


Figure 4. Aliskiren (Alisk) inhibits the migration of TNF- α -induced HASMC (A, B). It also reduced matrix metalloproteinase production (C) as well as gelatinolytic activity (D) in TNF- α -induced HASMC cultures. The results are expressed as the mean \pm SEM from three triplicate experiments. * means $p < 0.05$, compared with untreated control; # means $p < 0.05$, compared with TNF- α stimulated group without aliskiren treatment; scale bar, 75 μ m. Abbreviations are in Figure 3.

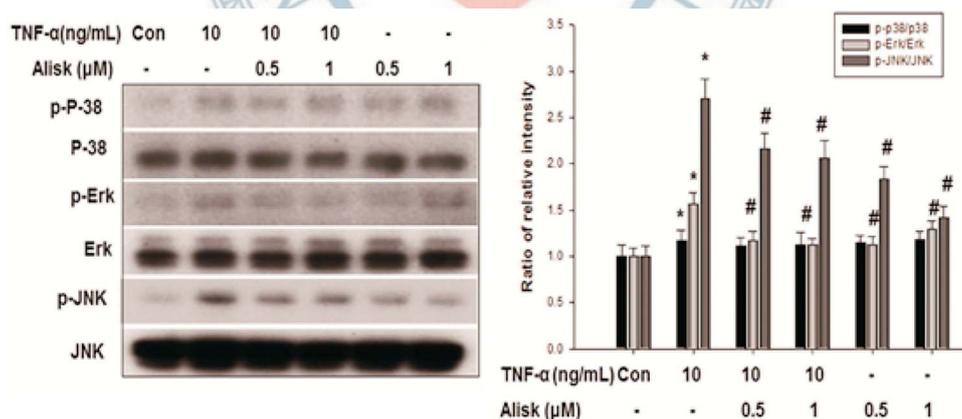


Figure 5. Aliskiren (Alisk) inhibits TNF- α stimulated p-ERK and P-JNK. * means $p < 0.05$, compared with untreated control; # means $p < 0.05$, compared with TNF- α stimulated group without aliskiren treatment. Abbreviations are in Figure 3.

tion in the urinary albumin-to-creatinine ratio of at least 50% from baseline.¹⁵

In animal studies, the production of intra-aortic acetylcholine-induced nitric oxide was significantly in-

creased, and vascular superoxide and peroxynitrite levels were both reduced in Watanabe heritable hyperlipidemic rabbits after aliskiren treatment. Furthermore, co-treatment with aliskiren and valsartan has additive

protective effects on endothelial function and atherosclerotic changes. The plaque area was significantly decreased by combination therapy compared with monotherapy with either drug in the aortic tissues.¹⁶

Wu et al.¹⁷ reported a reduction of atherosclerotic plaque development, neovessel formation, and increased collagen in an apolipoprotein-E knocked out mouse model. Martins-Oliveira et al.¹⁸ showed that losartan and aliskiren exerted the same anti-hypertensive effect, but only losartan prevented vascular remodeling and inhibited up-regulation of the expression of MMP-2.¹⁸

In the current study we showed that aliskiren reduced neointimal hyperplasia in hypercholesterolemic rabbits after balloon injury and the expression of MMPs in the neointima. The expression and activities of MMP-2 and -9 on TNF- α -treated human aortic SMCs was inhibited by aliskiren via the MAPK pathway.

Study limitations

In the current study, aliskiren had a direct effect on the expression of MMPs and neointima formation in hypercholesterolemic animals after vascular injury. However, the expression of pro-renin receptors in atheroma plaques, monocyte distribution, and the expression of other inflammatory markers in the neointima warrants further study.

CONCLUSIONS

Aliskiren inhibited expression of vascular matrix metalloproteinase in vivo and in vitro. In the future, we would like to investigate the clinical significance of renin inhibitors in diabetes.

CONFLICT OF INTEREST STATEMENT

None declared.

ACKNOWLEDGEMENT

We thank Novartis Taiwan for graciously providing the aliskiren as a gift. This study was supported, in part, by research grant VGH-V101C-174 from Taipei Veterans

General Hospital. This work was assisted in part by the Division of Experimental Surgery of the Department of Surgery at Taipei Veterans General Hospital.

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