主論文の要旨

Renin inhibition reduces atherosclerotic plaque neovessel formation and regresses advanced atherosclerotic plaques

（レニン阻害剤投与によるアテローム性動脈硬化プラークの進展ならびに新生血管に対する抑制効果及びその分子機序）

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【Introduction】

The renin-angiotensin system (RAS) plays a pivotal role in the pathogenesis of atherosclerosis-based cardiovascular disease. Angiotensin II (Ang II) is a major bioactive component of the RAS. Aliskiren, a direct renin inhibitor which reduces the formation of angiotensin I from angiotensinogen, suppresses Ang II biosynthesis at the first step of the RAS. A few experimental studies have reported the anti-atherosclerotic effects of aliskiren at the early stage of atherosclerosis. However, the vasculoprotective action of aliskiren's renin inhibition on advanced atherosclerosis and its underlying mechanisms are not yet understood.

TLR2 has been shown to play an important role in the progression of atherogenesis. Several lines of evidence indicate that RAS activation induces vascular inflammation through a TLRs-dependent signaling pathway; however, the precise mechanisms of the RAS and the TLR signaling pathways in the progression of atherosclerosis remain unknown. Neovascularization is associated with advanced atherosclerotic plaque growth. Angiogenic action stimulation has been closely linked to the Ang II/AT1R signaling pathway and protease activation (including cysteinyl cathepsins). However, the role of Ang II signaling in cathepsin activation-induced plaque neovessel formation and the mechanisms underlying the vasculoprotective action of the upstream inhibition of the RAS remain unclear, especially in atherogenesis at the advanced stage. To address these issues, we examined the effect of aliskiren-mediated RAS inhibition on the pathogenesis of advanced atherosclerosis in $ApoE^{-/-}$ mice, with a special focus on the plaque neovessel formation associated with TLR-dependent inflammation and cathepsin activation.

【Materials and Methods】

Animals and Treatment

4-wk-old $ApoE^{-/-}$ mice (male, C57BL/6 background) were fed a Western-type diet containing 21.00% fat from lard and 0.15% cholesterol for 8 wks, and the mice were randomly assigned to one of three groups and administered vehicle (control), hydralazine (25 mg mg/kg per day, in drinking water, Sigma-Aldrich; or aliskiren (25 mg/kg per day via a subcutaneous mini-pump, Novartis) for an additional 12 wks.

Histological and Immunohistochemical Staining and Morphometry

Mice were anesthetized by pentobarbital (i.p injection). The hearts with aortic roots were dissected and immersed in fixative for 24 h (4°C) and embedded in paraffin. Paraffin sections (4 μm) from the aortic roots were deparaffinized and stained with hematoxylin-eosin staining, Elastica van Gieson staining, and Picrosirius Red staining. For the immunohistochemical staining, the sections were incubated against macrophages and CD31, CatS, α-smooth muscle cell actin, and against monocyte chemotactic protein-1.

The positive areas for each stain were analyzed with BZ8000 analysis software (Keyence) or MetaMorph imaging analysis software (Molecular Devices).

HUVECs Small-Interfering RNA Transfection

HUVECs were transfected with TLR2 siRNA (siTLR2-I, siTLR2-II,) and CatS siRNA (siCatS, Sigma-Aldrich) for 48 hrs. Transfected cells were incubated with Ang II for 12 h and subjected to PCR assays.

Quantitative Real-time Polymerase Chain Reaction

We isolated total RNA from the aortic roots and the lysates from HUVECs with the use of an RNeasy® Micro kit (Qiagen) and subjected it to reverse transcription. The resulting cDNA was subjected to a quantitative real-time PCR analysis with the use of a Bio-Rad CFX96™ Real-Time PCR Detection System or Applied Biosystems Prism 7500HT sequence detection system (Applied Biosystems). We calculated the changes in gene expression by the $2^{-\Delta\Delta Ct}$ method and normalized the values to the levels of glyceraldehyde 3-phosphate dehydrogenase.

Western blotting analysis

Total extracted protein from aortic roots and HUVECs was separated with 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane, and incubated with primary antibodies against TLR2 and CatS. β-actin was loaded as control. The band intensitiy was analyzed by densitometry using Image J software.

Statistical Analysis

Data are expressed as means ± standard error of the mean. Student's t-test (for comparisons between two groups) and a one-way analysis of variance (ANOVA; for comparisons of three or more groups) followed by the Bonferroni post-hoc test were used for the statistical analyses. The HR and BP data were subjected to a two-way repeated-measures ANOVA and Bonferroni post hoc tests. SPSS software ver. 17.0 (SPSS, IL) was used. P-values <0.05 were considered significant.

【Results】

Aliskiren reduced the atherosclerotic plaque area and plaque neovessel density. It increased the plaque collagen and elastin contents, and reduced plasma angiotensin II levels and plaque macrophage infiltration and cathepsin S (CatS) protein. Aliskiren also decreased the levels of AT1R, gp91phox, TLR2, monocyte chemotactic protein-1, and CatS mRNAs in the aortic roots. Hydralazine had no beneficial vascular effects, although its administration resulted in the same degree of blood pressure reduction as aliskiren.

CatS deficiency mimicked the aliskiren-mediated vasculoprotective effect in the ApoE$^{-/-}$ mice, but aliskiren showed no further benefits in ApoE$^{-/-}$ CatS$^{-/-}$ mice. *In vitro*, TLR2 silencing reduced CatS gene expression induced by angiotensin II. Moreover, aliskiren or the inhibition of CatS impaired the endothelial cell angiogenic action *in vitro* or/and *ex vivo*.

【Discussion】

In this study, we have demonstrated that aliskiren has a vasculoprotective effect on advanced atherosclerosis. Ang II inhibition by renin inhibition with aliskiren not only lessened the progression of atherosclerotic lesions but also changed the composition of the vascular wall such that it contained more extracellular matrix protein (collagen and elastin), changes that predict greater stability of the atherosclerotic plaque. Renin inhibition also reduced the diet-induced intimal neovascularization in atherosclerotic plaques, which was accompanied by a reduced accumulation of macrophages and reduced expression of CatS associated with the reduction in TLR2 gene expression. More notably, we showed that CatS deficiency lessened diet-induced atherosclerotic plaque neovessel formation and growth, as well as the levels of TLR2 mRNA and macrophage infiltration in ApoE$^{-/-}$ mice. Our *in vitro* experiment also demonstrated Ang II induced CatS gene expression via the TLR2-mediated signaling pathway. Collectively, these findings suggest that the attenuation of neovascularization and ECM remodeling by renin inhibition via the inhibition of CatS expression represents a common mechanism for the reduction of diet-induced advanced atherosclerotic plaque growth and stability.

【Conclusion】

Renin inhibition appears to inhibit advanced plaque neovessel formation in ApoE$^{-/-}$ mice and to decrease the vascular inflammatory action and extracellular matrix degradation, partly by reducing AT1R/TLR2-mediated CatS activation and activity, thus regressing advanced atherosclerosis.