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Aliskiren attenuates the effects of interleukin-6 on endothelial nitric oxide synthase and caveolin-1 in human aortic endothelial cells

Ming-Jui Hung*, Yu-Cheng Kao, Chun-Tai Mao, Tien-Hsin Chen, Wei-Siang Chen

Section of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Keelung City, Taiwan

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ABSTRACT

Renin inhibitors enhance endothelial nitric oxide synthase (eNOS) bioavailability and have protective effects on endothelial function and atherosclerotic changes. This study was designed to investigate whether aliskiren attenuates the effects of interleukin-6 (IL-6) on eNOS and the eNOS-caveolin-1 interaction in human aortic endothelial cells (HAECs). In this study, we examined the effects of pretreatment with aliskiren on the changes of IL-6-induced expression and activation of eNOS and caveolin-1 in cultured HAECs. IL-6 inhibited and aliskiren increased the phosphorylation of eNOS at Ser1177; however, eNOS protein and mRNA expression were not changed. Pretreatment with aliskiren attenuated the inhibitory effects of IL-6 on eNOS phosphorylation and nitric oxide production. IL-6 increased the phosphorylation of caveolin-1 at Tyr14 without affecting the caveolin-1 protein and mRNA expression. Pretreatment with aliskiren attenuated the effects of IL-6 on caveolin-1 phosphorylation. The binding of eNOS and caveolin-1, as determined by a co-immunoprecipitation assay, was increased by IL-6 treatment and decreased by aliskiren pretreatment. Furthermore, treatment with short interfering RNA of the extracellular signal-regulated kinase gene reversed the effects of IL-6 and aliskiren on eNOS and caveolin-1. In conclusion, aliskiren attenuates the inhibitory effects of IL-6 on eNOS phosphorylation and nitric oxide production and IL-6 induced caveolin-1 phosphorylation. In addition, aliskiren reverses the effects of IL-6 on the eNOS-caveolin-1 interaction.

1. Introduction

The exposure of endothelial cells to pro-inflammatory cytokines leads to the expression of cell surface adhesion molecules and impairs endothelium-dependent vascular relaxation [1]. These alterations in endothelial cell function, collectively termed “endothelial activation,” promote acute events in atherosclerotic vascular disease. Endothelial nitric oxide synthase (eNOS) is the only nitric oxide synthase isoform dually acylated by the fatty acids myristate and palmitate [2], thereby strongly supporting a preferential membrane location of the enzyme. eNOS is quantitatively associated with caveolin-1 in endothelial cells [3]. Acute changes in pressure or shear stress can dissociate eNOS from caveolin-1 through a calcium-calmodulin dependent pathway and lead to vasodilation [4]. Interleukin-6 (IL-6) inhibited eNOS activation and increased eNOS binding to the prolonged half-life of caveolin-1, i.e. stabilized caveolin-1 [5], indicating that inflammation impairs endothelial function, an early step in atherosclerotic cardiovascular disease.

Angiotensin II, the effector hormone of the renin-angiotensin system (and eventually aldosterone), exerts several effects on various vessel wall cell components functioning as a growth, migration, prothrombotic, and proinflammatory factor [6–8]. In a mouse model of atherosclerosis, angiotensin II induced the progression of atherosclerosis and mediated plaque vulnerability beyond its effect on blood pressure [9]. Secretion of renin is not only the first but also the rate-limiting step in renin-angiotensin-aldosterone system cascade. Renin-angiotensin-aldosterone system could be blocked by pharmacological agents at various sites. Both angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are the incomplete blockade of renin-angiotensin-aldosterone system with substantial rise of renin and angiotensin peptides. Inhibition of renin would block the renin-angiotensin-aldosterone system at the highest level and at its origin. The renin inhibitor aliskiren enhanced eNOS bioavailability through a reduction of eNOS uncoupling and had protective effects

* Corresponding author. Section of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital, 222 Maijin Road, Keelung City, 20401, Taiwan.
E-mail address: hmj1447@cgmh.org.tw (M.-J. Hung).

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on endothelial function and atherosclerotic change [10]. Thus, treatment with a renin inhibitor could have the potential to reduce atherosclerosis.

In this study, we hypothesized that aliskiren attenuated the effects of IL-6 on the deactivation of eNOS and the interaction of caveolin-1 and eNOS in human aortic endothelial cells (HAECs). To test this hypothesis, we examined the effects of aliskiren and IL-6 on eNOS, caveolin-1, as well as their interactions in cultured HAECs.

2. Materials and methods

2.1. Reagents

IL-6 was purchased from Sigma (St. Louis, MO, USA). Aliskiren (0.1–1000 nM) was provided by Roche Molecular Biochemicals (Norvatis Pharmaceuticals, Basel, Switzerland). We used aliskiren, a renin-inhibitor, instead of angiotensin II receptor antagonists or angiotensin-converting enzyme inhibitors in this study because of its direct inhibition of the renin-angiotensin-aldosterone system.

2.2. Cell culture

HAECs were purchased from Cell Applications Inc. and were maintained in phenol-red-free Medium 199 (GIBCO; Invitrogen, Carlsbad, California, USA) supplemented with 20% charcoal/dextran-treated fetal bovine serum (GIBCO), 20 mmol/l HEPES, and 100 μg/ml endothelial cell growth factor (Collaborative Research Inc., New York, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% room air. Trypsin-EDTA was used for subculturing. All media were supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin. The medium was refreshed every third day. Cells at 80%–90% confluence and between passages 3 and 5 were used in all of the experiments. Based on prior studies of aliskiren [11], eNOS, and caveolin-1 [5], cells were treated with aliskiren for 1 h and/or IL-6 at concentrations of 10 ng/ml for 6 h.

2.3. Transfection and lipofectamine assay

HAECs were transfected with short interference RNA (siRNA) of extracellular signal-regulated kinase (ERK) using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, California, USA). Twenty-four hours following transfection, the cells were incubated with or without aliskiren 100 nM for 1 h followed by IL-6 10 ng/ml for 6 h. At that time, cells were collected and lysed for eNOS and caveolin-1 analysis.

2.4. Nitric oxide and renin measurement

Nitric oxide measurement was performed using a nitrate/nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA), which detects nitric oxide by measuring nitrate and nitrite. This kit is based on the colorimetric change at absorbance of 415 nm, which occurs when and 1(H)-naphthotriazole is added to the byproduct of reaction between 2,3-diaminonaphthalene and nitrite. Further nitric oxide detection was performed using a diaminofluorescein method (Molecular Probes, Inc. Leiden, Netherlands). Renin activity measurement was performed using a sandwich enzyme immunoassay kit (R&D Systems, Inc. Minneapolis, Minnesota).

2.5. Western immunoblots

Cells were lysed in lysis buffer (50-mM Tris-HCl, 150-mM NaCl, 1-mM ethyleneglycoltetraacetic acid (EGTA), 1% Nonidet P-40 [NP-40], 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 1 mg/ml aprotinin, and leupeptin, pH 7.4) on ice for 20 min. The protein concentrations of the cell lysates were determined. Equal amounts of protein were loaded in each lane of a sodium dodecyl sulfate-polyacrylamide gel and resolved at 90 V for 2 h. Gels were transferred onto polyvinylidene difluoride membranes (Bio-Rad, Foster City, California, USA) at 80 V for 1.5 h at room temperature. The membranes were then blocked in 5% bovine serum albumin and 1% goat serum in phosphate-buffered saline with 0.1% Tween 20. Blots were incubated in anti-eNOS (1:500), anti-phospho-eNOS (Ser1177, 1:500), anti-caveolin-1 (1:2000), anti-serine/threonine kinase-protein kinase B (Akt, 1:2000), anti-phospho-Akt (Ser473 and Thr308, 1:500), anti-ERK1/2 (1:1000), anti-phospho-ERK1/2 (Thr202/Tyr204, 1:1000), and anti-phospho-caveolin-1 (Tyr14, 1:800) then rinsed three times followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies for 60 min. After washing, the blots were developed with enhanced chemiluminescence on Hyperfilm (Amersham Biosciences, Arlington Heights, Illinois, USA). Anti-α-tubulin antibody was from Novus Biologicals (Littleton, Colorado, USA). All other antibodies were obtained from Cell Signaling Technology (Irvine, California, USA). Densitometric analysis was performed directly from the membrane using a Bio-Rad Molecular Imager system.

2.6. Co-immunoprecipitation

Cells were lysed in the co-immunoprecipitation buffer (20 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM phenylmethylsulfonylfluoride, 0.5% protease inhibitor cocktail, pH7.5) on ice for 20 min. The 50 μl Qbeads-Protein A (Magqu) were incubated with anti-caveolin-1 (1:50) or anti-eNOS (1:50) antibody at room temperature with rocking for 1 h. After binding of antibody, 100 μl lysate (50 μg protein) was added with incubation for 1-h at room temperature. Twenty-five μl elution buffer (0.1 M glycine-HCl, pH 2.0) was added and gently mixed with the Qbeads-antibody-antigen complex by pipetting. The immune complexes were resolved on sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose. The blots were blocked with PBST (phosphate buffered saline with 0.1% Tween 20). Blots were developed with diluted antibodies for eNOS (1:300) and caveolin-1 (1:2000), followed by horseradish peroxidase-conjugated goat anti-rabbit (diluted 1:5000). The blots were detected by enhanced chemiluminescence according to the manufacturer's instructions. Band intensities of the autoluminographs were quantitated by densitometry.

2.7. Reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time polymerase chain reaction (PCR)

Total RNA was isolated by cell lysis in guanidinium isothiocyanate buffer followed by a single step phenol-chloroform-isoamyl alcohol extraction. Briefly, the HAOECs were harvested and lysed in solution D containing 4-M guanidium isothiocyanate, 25-mM sodium citrate (pH 7.0), 0.5% sodium sarcosine, and 0.1-M β-mercaptoethanol. Sequentially, 1/10 volume of 2-M sodium acetate (pH 4.0), one volume of phenol, and 1/5 volume of chloroform-isoamyl alcohol (49:1, v:v) were added to the homogenate. After vigorous vortexing for 30 s, the solution was centrifuged at 10,000 × *g* for 15 min at 4 °C. RNA in the aqueous phase was precipitated by the addition of 0.5 ml of isopropanol. One μg of total RNA was reverse-transcribed into cDNA by incubating with 200 units of reverse transcriptase in 20 μl of reaction buffer containing 0.25 μg of random primers and 0.8-mM dNTPs at 42 °C for one hour. Two μl of the cDNA were used as templates for the PCR reaction. The PCR was performed in buffer containing 10-mM Tris, pH 8.3, 50-mM KCl, 1.5-mM $MgCl_2$, 0.2-mM dNTPs, 1-μM of each primer, and 5 units Taq