Dynamic Observation of Mechanically-Injured Mouse Femoral Artery Reveals an Antiinflammatory Effect of Renin Inhibitor

Jun Ino, Chiari Kojima, Mizuko Osaka, Kosaku Nitta, Masayuki Yoshida

**Objective**—The renin-angiotensin-aldosterone system (RAS) plays a central role in atherosclerosis. To investigate the effects of a direct renin inhibitor aliskiren on vascular inflammation, we conducted leukocyte adhesion assays in vivo and in vitro using a novel real-time imaging system.

**Methods and Results**—Aliskiren (10 mg/kg/d) or PBS was administered to C57BL/6 mice (6–7 weeks of age; Oriental Yeast, Tokyo, Japan) for 2 weeks via an osmotic pump. Blood pressure was not significantly changed in the 2 groups throughout the experimental period. A perivascular cuff injury was then introduced to the femoral artery and real-time intravitral microscopic observation was conducted 24 hours after injury. The number of adherent leukocytes was elevated in the injured mice without aliskiren (43.8 ± 9.3/10^-2 mm²), whereas that was significantly reduced in the mice with aliskiren (18.4 ± 4.4, P < 0.05). Treatment of human umbilical vein endothelial cells (HUVECs) with aliskiren significantly reduced the adhesion of THP-1 cells to TNF-α-activated HUVECs (P < 0.05). Interestingly, TNF-α-induced renin activity and angiotensin II production in HUVECs were also blunted by aliskiren. Furthermore, exogenous renin and angiotensin II abrogated the aliskiren-mediated reduction of THP-1 cell adhesion to HUVECs.

**Conclusions**—Our in vivo and in vitro findings indicate a pivotal role for renin inhibition in vascular inflammation independent of blood pressure. (Arterioscler Thromb Vasc Biol. 2009;29:1858-1863.)

**Key Words:** atherosclerosis  ■ renin-angiotensin-aldosterone system  ■ renin inhibition  ■ endothelium

The renin-angiotensin-aldosterone system (RAS) has been recognized as one of the key mechanisms involved in various vascular diseases including atherosclerosis. Recent reports have also demonstrated that production of RAS in extrarenal tissues exerts critical biological effects, whereas regulation of local RAS contributes to the pathophysiological condition of endothelial cells and smooth muscle cells. Therefore, modulation of local as well as systemic RAS has emerged as a therapeutic concept for cardiovascular diseases.

Angiotensin (Ang) II, a potent vasoconstrictive peptide, plays a crucial role in atherosclerosis via local activation of mitogen-activated protein kinases (MAPK). In addition, we previously found that Ang II–related oxidative stress in leukocytes induces leukocyte recruitment in vivo. However, the continued presence of other bioactive angiotensin-related peptides has made it difficult to elucidate the contributions of Ang II in these processes.

Renin, a rate-limiting enzyme for Ang II formation, exerts various inflammatory reactions, including activation of thrombogenic molecules and intracellular signaling proteins independent of local Ang II, which are mediated via the recently characterized renin receptor (RR). Recently aliskiren, a direct renin inhibitor, has been shown to modulate vascular diseases such as hypertension, experimental atherosclerosis, and diabetic nephropathy via RAS dependent or independent pathway. Although local RAS are important in those processes, the importance of renin inhibition in vascular inflammation has not been elucidated.

In the present study, we focused on the effects of aliskiren on leukocyte-endothelial interaction, a crucial step in vascular inflammation. We used intravitral microscopy (IVM) to visualize acute phase leukocyte recruitment in mouse femoral arteries injured by perivascular cuff placement and also examined the responsible molecular mechanisms in vitro.

**Materials and Methods**

The Materials and Methods section detailing the techniques and procedures used in the present study is available as a supplemental materials (available online at http://atvb.ahajournals.org).

**In Vivo Experiments**

Aliskiren diluted in PBS or PBS alone was administered to C57BL/6 mice via an osmotic pump for 2 weeks. On day 13, a perivascular cuff was placed on the right femoral artery, and on day 14, the mice were injected via the left femoral vein with fluorescent labeled leukocytes. Immediately after the injection, the injured femoral
artery was examined with an epifluorescent microscope and observed endothelial-leukocyte interactions images captured via an ultrasensitive camera.

Adhesion Assay Under Flow

Human umbilical vein endothelial cells (HUVECs) were stimulated with or without tumor necrosis factor α (TNF-α) and aliskiren on coverslips, then positioned in a flow chamber. THP-1 cells were perfused and drawn through the chamber at a controlled flow rate to generate a shear stress.

Western Blot Analysis and Quantitative RT-PCR Analysis

Lysate or mRNA was prepared from HUVECs in vitro or injured specimen in vivo and conducted after immunoblotting or RT-PCR analysis.

Reactive Oxidative Stress Generation Analysis

HUVECs, stimulated with TNF-α and aliskiren were incubated with reactive oxidative stress (ROS)-detecting probe, CM-H₂DCFDA, according to the manufacturers’ protocol. Fluorescence intensity was evaluated using FACS caliber and CellQuest software.

Results

Aliskiren Reduced Leukocyte Recruitment After Injury In Vivo

To examine the antiinflammatory effects of aliskiren in vivo, we administered PBS or aliskiren at a dose of 3, 10, 25, or 50 mg/kg/d to C57BL/6 mice for 2 weeks. As shown in supplemental Figure I, treatment with aliskiren at 25 or 50 mg/kg/d significantly decreased blood pressure after 2 weeks. In contrast, lower doses, such as 3 and 10 mg/kg/d, did not alter blood pressure. Heart rate and body weight were not significantly altered among the groups during the 2-week experimental period (data not shown).

A perivascular cuff was placed on the femoral artery of mice on day 13 of PBS or aliskiren treatment and IVM observations were performed the following day. The representative snapshots (Figure 1A through 1C) and video images (supplemental Movie) revealed that treatment with aliskiren reduced leukocyte recruitment to the injured arteries. Furthermore, image analysis (Figure 1D) showed that cuff placement significantly increased the number of adherent leukocytes (43.8±9.3×10² mm²), which was dose-dependently reduced by aliskiren. To separate the effect of aliskiren on blood pressure from that on inflammation, we used a lower dose (10 mg/kg/d) in the following experiments. We found that plasma renin activity and Ang II concentration were significantly decreased after aliskiren administration, which confirmed its efficacy in mice under the present study conditions (supplemental Figure II).

Aliskiren Reduced Leukocyte–Endothelial Interaction In Vitro

To explore the molecular mechanisms of the antiadhesive action of aliskiren, we conducted a leukocyte-endothelial adhesion assay in vitro under a flow condition. Following our finding of TNF-α upregulation in the injured arteries (supplemental Figure III), we stimulated HUVECs with TNF-α to mimic that effect in vitro. TNF-α-induced THP-1 adhesion to HUVECs was significantly reduced by aliskiren in a dose-dependent manner (Figure 2A) and reached a plateau after 4 hours (Figure 2B). In addition, Western blotting (Figure 2A) and real-time PCR (supplemental Figure IV) analyses revealed that TNF-α-induced upregulation of E-selectin, ICAM-1, and VCAM-1 in HUVECs was attenuated by aliskiren, as was TNF-α-induced activation of ERK, JNK, p-38 (Figure 3B) and NF-κB (supplemental Figure VA). Considering the relatively short time course of TNF-α stimulation (0.5 hour), the antiinflammatory effects of aliskiren may involve an RAS-independent signaling pathway. Thus, we compared the effects of aliskiren and an angiotensin
receptor blocker (ARB), valsartan, as well as their combination. Aliskiren, but not valsartan, reduced JNK activation at 0.5 hour after TNF-α-stimulation (Figure 3C). In contrast, aliskiren and valsartan equally reduced JNK activation after 4 hours. A combination of both reduced JNK activation at 0.5 hour to a level comparable to aliskiren, and at 4 hours to a level comparable to aliskiren and valsartan administered separately.

**Aliskiren Reduced Renin- and Ang II–Dependent Inflammation In Vitro**

We also evaluated the involvement of RAS in vitro. First, we measured renin activity and Ang II concentration in the culture medium of HUVECs. TNF-α increased local renin activity and Ang II production in the medium, both of which were decreased by aliskiren (supplemental Figure VI). In contrast, the expression and activity of angiotensin converting enzyme (ACE) in HUVECs were not changed by TNF-α or aliskiren (supplemental Figures VII and VIII). Because local RAS has been shown to play a role in vascular inflammation, we investigated whether the antiadhesive effect of aliskiren is mediated through inhibition of local RAS. Though exogenous renin and Ang II did not enhance THP-1 adhesion to TNF-α-activated HUVECs, they restored aliskiren-mediated antiadhesive effects (supplemental Figure IXA). Further, they were able to enhance THP-1 adhesion to minimally activated HUVECs (TNF-α at 0.1 ng/mL; supplemental Figure IXB). Exogenous renin enhanced expressions of E-selectin, ICAM-1, and VCAM-1 (Figure 4A). It also restored aliskiren-mediated downregulation of adhesion molecules and inactivation of ERK, JNK, p38 (Figure 4B), and NF-κB (supplemental Figure VB).

**Aliskiren Attenuated TNF-α–Induced Oxidative Stress in HUVECs**

To investigate the potential modulation of oxidative stress by aliskiren, the amount of oxidative stress was evaluated by examining the fluorescent intensity of CM-H2DCFDA in HUVECs by flow cytometry. Aliskiren inhibited TNF-α–mediated oxidative stress (Figure 5A and supplemental Figure X) along with a reduction in TNF-α-induced gp91phox expression, a component of NADPH oxidase in HUVECs (Figure 5B). Furthermore, TNF-α–mediated reduction of eNOS expression was recovered by aliskiren (Figure 5C).

**Figure 3.** Effects of aliskiren on adhesion molecules, MAPK, and NF-κB in HUVECs. A, HUVECs were activated with TNF-α (0.1, 1, 10 ng/mL) in the presence or absence of aliskiren (10⁻⁶ mol/L) for 4 hours. The expressions of E-selectin, ICAM-1, and VCAM-1 were evaluated by Western blotting analysis as described in Materials and Methods. An average of 3 independent blots is presented. Values are shown as the mean±SEM. *P<0.01 vs TNF-α (−), **P<0.05 vs TNF-α (+) aliskiren (−) (at comparable TNF-α concentration). B, HUVECs were activated with TNF-α (1 ng/mL) in the presence or absence of aliskiren (10⁻⁷, 10⁻⁶ mol/L) for 0.5 hour, and Western blotting analysis was performed as described in Materials and Methods. The expressions of phosphorylated ERK, JNK, and p38 were normalized with those of their respective total proteins. Values are shown as the mean±SEM of 3 independent experiments and presented as bars below each blot. *P<0.05 vs TNF-α (−), **P<0.05 vs TNF-α (+) aliskiren (−). C, HUVECs were activated with TNF-α (1 ng/mL) in the presence or absence of aliskiren (10⁻⁶ mol/L) or valsartan (10⁻⁴ mol/L) for 0.5 or 4 hours, and the expression of phosphorylated JNK was detected by Western blotting as described in Materials and Methods. *P<0.01 vs TNF-α (−), **P<0.05 vs TNF-α (+) aliskiren (−) for 0.5 hour, #P<0.05 vs TNF-α (+) aliskiren (−) for 4 hours. Alisk indicates aliskiren; Val, valsartan; Comb, combination of aliskiren and valsartan.
Aliskiren Stabilized Local Inflammation In Vivo

To explore the molecular link between renin inhibition and inflammation in vivo, we examined the expressions of adhesion molecules and representative signaling molecules, such as MAPK and NF-κB, in the injured artery specimens. As shown in Figure 6A and 6B, perivascular cuff injury induced upregulation of E-selectin, ICAM-1, VCAM-1, and gp91phox in the femoral arteries, which was reduced by treatment with aliskiren. Moreover, perivascular injury also activated the expressions of ERK, JNK, p38, and NF-κB, which were attenuated by aliskiren (Figure 6C and supplemental Figure VC). In addition, aliskiren reduced and increased eNOS expression in the injured arteries (Figure 6D), which was similar to our finding in vitro.

Discussion

Initiation of vascular disorders contains an inflammatory component characterized by leukocyte-endothelial interaction, in which orchestration of various adhesion molecules regulates the entire process. Herein, we focused on the effects of direct renin inhibition by aliskiren on leukocyte-endothelial interaction in vivo and in vitro.

Our results demonstrated that renin inhibition by aliskiren significantly reduced the number of leukocytes that adhered to cuff-injured femoral arteries in mice. Polyethylene cuff placement around the femoral artery has been shown to induce vascular inflammation accompanied by vascular remodeling. Although proliferation of smooth muscle cells and neointimal formation has been repeatedly documented in studies that used this injury model, acute phase vascular inflammation such as leukocyte recruitment to the injured artery has not been demonstrated. In the present study, we confirmed that perivascular cuff placement significantly induces leukocyte adhesion in the femoral artery. To our knowledge, this is the first real-time observation of leukocyte recruitment after perivascular injury in vivo as well as its blockade by renin inhibition. A reduction in leukocyte recruitment in vivo after aliskiren administration was achieved at a dose that showed no significant changes in blood pressure. Moreover, our in vitro experiments were conducted in HUVECs were determined by quantitative real-time PCR. The average of 3 independent experiments is shown. Values are shown as the mean±SEM. *P<0.05 vs TNF-α (−), **P<0.05 vs TNF-α (+) aliskiren (−), †P<0.01 vs TNF-α (+) aliskiren (−).
with cultured endothelial cells that were not exposed to high atmospheric pressure. Therefore, the observed effects of aliskiren were not a direct consequence of its ability to lower blood pressure. In addition, we observed that aliskiren reduced vascular remodeling at 28 weeks after cuff injury (supplemental Figure XI), which confirmed the antiatherosclerotic effect of renin inhibition.

Previous studies including ours have suggested a dominant role for adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, in vascular inflammation. In the present study, we observed upregulation of these adhesion molecules in the injured arteries, each of which was reduced by aliskiren. We also documented the involvement of ERK, JNK, p38, and NF-κB–dependent pathway in injury-induced leukocyte recruitment in vivo. Moreover, the cuff injury caused downregulation of eNOS expression and upregulation of gp91phox in the injured arteries. These results show a causative role for oxidative stress and its stabilization by aliskiren in the present experimental model. Recent studies have suggested that Ang II induces inflammation and atherosclerosis via local activation of ERK, JNK, p38, and NF-κB–dependent pathway in injury-induced leukocyte recruitment in vivo. In addition, oxidative stress induced by Ang II causes activation of NF-κB29 and subsequent induction of E-selectin. ICAM-1,28 and VCAM-1.29 Although the precise mechanisms are unclear at present, reduction in oxidative stress and stabilization of MAPK and NF-κB may play dominant roles in aliskiren-mediated antiinflammatory effects in vivo. Exogenous renin and Ang II cancelled antiinflammatory actions of aliskiren including expressions of adhesion molecules, activation of MAPK and NFKB, and generation of ROS in activated HUVECs. Moreover, we observed that renin and Ang II enhanced THP-1 adhesion to minimally activated HUVECs with TNF-α at 0.1 ng/mL (supplemental Figure IXB). These results suggest that a magnitude of HUVEC stimulation by renin and Ang II is smaller than that by TNF-α at 1 ng/mL. Thus exogenous renin and Ang II failed to enhance THP-1 adhesion to HUVECs activated with 1 ng/mL TNF-α. Interestingly, the inhibitory effects of aliskiren on MAPK and NF-κB became apparent at 0.5 hour after TNF-α stimulation. Because TNF-α treatment increased Ang II production within 4 hours after stimulation (supplemental Figure XII), aliskiren may directly block TNF-α–dependent signaling in the absence of an Ang II–mediated RAS pathway. In fact, we confirmed that an ARB failed to inhibit JNK activation at 0.5 hour after TNF-α activation (Figure 3C). In contrast, both aliskiren and the ARB reduced JNK activation at 4 hours after activation of TNF-α. Therefore, we speculate that aliskiren not only reduces the activity of the local RAS dependent pathway, but also directly inhibits TNF-α–dependent inflammation in the absence of RAS (see supplemental Figure XIII). This supports the notion of combined effects of an ARB and aliskiren in hypertension and atherosclerosis via modulation of RAS-dependent and -independent pathways.

Recent reports have pointed out a potential role for RR in RAS-independent signaling by renin. In addition, Feldman et al recently described the involvement of RR in aliskiren-mediated beneficial effects in diabetic nephropathy rat. The contribution of an RR-dependent pathway in aliskiren-mediated modulation of vascular inflammation requires further investigation.

As previously reported, leukocyte adhesion accelerates MAPK, NF-κB, and ROS generation in leukocytes, leading to the development of a viscous cycle of inflammation. Thus, we consider that aliskiren may exert a clinical advantage by blocking exaggerated inflammation after vascular injury via modulation of the inflammatory cascade in leukocytes. In fact, recent studies have reported effects of RAS inhibition in monocytes. We intend to focus on the potential effects of aliskiren on leukocytes in a future investigation.

Conclusion

Renin inhibition by aliskiren profoundly reduced leukocyte recruitment in perivascular cuff injury-induced acute vascular inflammation independent of blood pressure. The underlying mechanisms seemed to involve a reduction in adhesion molecules, MAPK, and NF-κB via RAS-dependent and -independent pathways.

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Disclosures
None.

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Supplement Materials

Dynamic Observation of Mechanically-injured Mouse Femoral Artery Reveals an Anti-inflammatory Effect of Renin Inhibitor

Supplemental methods

Reagent and Cell Culture

Aliskiren (SPP-100) and valsartan were kindly provided by Novartis International AG (Basel, Switzerland).

The reagents and antibodies used in the present study are as follows: Phosphate Buffered Saline (PBS), RPMI 1640 medium were from Sigma-Aldrich (St.Louis, MO), anti-E-selectin, anti-phosphorylated ERK, anti-ERK, anti-eNOS, anti-phosphorylated NF-κB, anti-NF-κB, anti-IκB were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-ICAM-1, anti-phosphorylated JNK, anti-phosphorylated p38, anti-JNK, and anti-p38 were from Cell Signaling Technology Inc. (Danvers, MA), anti-VCAM-1, and tumor necrosis factor-α (TNF-α) were from R&D Systems (Minneapolis, MN), and anti-ACE were from Chemicon International (Temecula, CA). Human umbilical vein endothelial cells (HUVEC) were isolated from normal term-umbilical vein and established in culture as previously described. Primary cultured were serially passaged (<1:3 split ratio) and maintained in medium 199 buffered with 25 mmol/L of RPMI1640 and supplemented with 20 % fetal bovine serum (FBS), endothelial cell growth factor (10 µg/ml), 100 U/ml penicillin and 100 µg/ml streptomycin, and porcine intestinal heparin (50 µg/ml). Cells were grown in a humidified incubator at 5 % CO2 and 37 °C. THP-1 cells were obtained from AmericanType Culture Collection and grown in RPMI1640 medium containing 10 % FBS, 100 U/ml of penicillin,100 µg/ml of streptomycin, and 2 mM L-glutamine.

In vivo experiments
Male C57BL/6 mice (6-7 weeks of age; Oriental Yeast, Tokyo, Japan) were fed a standard diet (CLEA Japan, Tokyo, Japan), and food and water were provided ad libitum. The experiments adhered to the American Physiological Society document “Guiding Principles in the Care and Use of Animals” and were approved by the Ethical Committee for Animal Experimentation of Tokyo Medical and Dental University.

Either aliskiren (3, 10, 25, 50 mg/kg/day) diluted in PBS (n=6) or PBS alone (n=7) was administered to C57BL/6 mice via an osmotic pump (Alzet model 2001, Durect Corp, Cupertino, CA) from day 0, which was maintained until day 14. On day 13, a perivascular cuff was placed on the right femoral artery to create an injury, and intravital microscopic (IVM) analysis of the injured femoral artery was conducted on day 14. Blood pressure (BP) and heart rate (HR) were measured through a tail cuff using a noninvasive automatic sphygmomanometer (BP-98A, Softron, Tokyo, Japan), and body weight (BW) was measured with a Sartorius L610 (Sartorius AG, Göttingen, Germany). These parameters were measured on days 0, 7 and 14. The detailed protocol of IVM has been described previously in detail. Briefly, the right femoral artery was exposed by blunt dissection. A cuff was carefully placed around the femoral artery to cover a 1 cm portion toward the iliac artery. IVM analysis was carried out 24 hours after cuff placement. The mice were anesthetized by pentobarbital sodium, and a catheter was placed in the contra-lateral femoral vein, while they were intubated and ventilated to maintain a normal acid-base balance. Rectal temperature was kept at 37°C with a heating pad and an infrared heat lamp. The exposed tissue was superfused with a thermostatic (37°C) bicarbonate-buffered saline solution. The mice were injected via the left femoral vein with rhodamine 6G chloride (Molecular Probes; 0.3 mg/kg in 200-300 µl of PBS) labeled leukocytes in vivo. Immediately after the injection, the injured femoral artery was examined with an epifluorescent microscope (model BX51WI, Olympus, Tokyo, Japan) and images were directly captured to a personal computer via an ultrasensitive charge-coupled device camera (Cool SNAP HQ, Olympus).

**Adhesion Assay under Flow**
The protocol of the adhesion assay under a flow condition has been described in detail previously. In brief, HUVEC monolayers were stimulated with or without tumor necrosis factor alpha (TNF-α) (1 ng/ml) for 4 hours and aliskiren for the indicated concentrations and periods on coverslips, then positioned in a flow chamber mounted on an inverted microscope (Nikon, Tokyo, Japan). THP-1 cells (1×10^6/ml) were prepared and resuspended with perfusion medium (PBS containing 0.2% human serum albumin), then perfused and drawn through the chamber with a syringe pump (PHD2000; Harvard Apparatus Inc., Holliston, MA) for 10 minutes at a controlled flow rate to generate a shear stress of 1.0 dyne/cm². The entire period of perfusion was recorded by videotape, and then transferred to a personal computer for image analysis to determine the number of adherent cells on HUVEC monolayers in 15 randomly selected 20× microscope fields.

**Western Blot Analysis**

*In vivo*, femoral artery was harvested from each group of mice immediately after IVM, fixed in liquid nitrogen and homogenized in ice with lysis buffer (20 nmol/L of Tris-Cl, 2 mmol/L of EDTA, 0.5 mmol/L of EGTA, 1 mmol/L of phenylmethylsulfonyl fluoride, 25 µg of leupeptine per ml, 0.1 mg of aprotinin per ml and 0.33 M sucrose). In *vitro*, HUVEC treated with TNF-α (1 ng/ml) with or without Aliskiren under indicated concentrations for 0.5 or 4 hours were washed and scraped with PBS, followed by sonicated with lysis buffer (1 % Triton X-100 in PHEM buffer proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and then they were centrifuged to recover the clear cell lysate. Western blotting (WB) was performed using previous described techniques. In brief, the samples (1~4×10^{-5} g per lane, differentiated by tissue and primary antibody) were resuspended in sample buffer, boiled (2 min), subjected to SDS-PAGE, and transfer electrophoresis. The transblots were probed with primary antibodies as described above followed by horse radish-labeled secondary antibodies (donkey anti-rabbit, 1:5,000; Amersham Pharmacia, Piscataway, NJ) and detected by chemiluminescence detection reagents (ECL advance or ECL, Amersham Pharmacia). The expressions of phosphorylated
proteins were normalized with those of total proteins and total proteins were compared with those of β-actin, respectively.

**Quantitative RT-PCR analysis**

Adhesion molecules, subunits of NADPH oxidase and RAS components mRNA expression in mice or cultured HUVEC was verified by quantitative RT-PCR. Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol after removal of injured artery *in vivo* and TNF-α (1 ng/ml) stimulation on HUVEC with or without Aliskiren (10⁻⁷ M or 10⁻⁶ M) for 1 hour *in vitro*. The complement DNA was synthesized from 1~3 µg of total RNA using Oligo (dT)₂₀ primers and Superscript III (Invitrogen, San Diego, CA). Quantitative PCR was performed applying a SYBR Premix Ex Taq™ (Takara-Bio Inc., Shiga, Japan) and run on a Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc.) using the following human- and mouse-pacific primer pairs (Supplemental Table). Target gene mRNA expression was normalized to β-actin mRNA expression, and the relative amounts of all of the mRNAs were calculated using the comparative Ct (threshold cycle) method ⁹.

**Reactive Oxidative Stress (ROS) generation analysis *in vitro***

Oxidative stress in HUVEC was quantitated using 5-(and-6)-chloromethyl-2', 7'-dichloro-dihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Invitrogen, San Diego, CA). HUVEC, stimulated with or without TNF-α (1 ng/ml) and aliskiren (10⁻⁶ M) for 2 hours, were incubated with CM-H₂DCFDA according to the manufacturers’ protocol. Fluorescence intensity was observed using an inverted fluorescence tissue culture microscope (Olympus IX70, Olympus Corp., Tokyo, Japan).

Moreover, to evaluate the intensity of ROS generation *in vitro*, HUVEC treated with TNF-α (1 ng/ml) with or without Aliskiren (10⁻⁶ M) for 2h were washed and scraped with buffer (HBSS(-) + 5 mM EDTA + 4mM EGTA), and washed with RPMI 1640 medium containing 5% fetal bovine serum, and then they were incubated with CM-H₂DCFDA according to the manufacturers’ protocol. The fluorescence
intensity in HUVEC was detected from 10000 cell-fraction using FACS caliber (BD Biosciences, San Jose, CA) at 580 nm, and the data was analyzed by CellQuest software (Becton Dickinson).

Measurement of RAS Components in vivo

Mice plasma was collected immediately after intravitreal microscopy analysis. Renin activity in mice were measured using a Renin activity assay kit (AnaSpec Inc., San Jose, CA). The value for mean fluorescence for each condition is expressed as a proportion to the control [injury (-) aliskiren (-)].

Ang II concentration in mice were measured using Ang II ELISA kit (Phoenix Pharmaceuticals) according to the manufacturer’s protocol.

Measurement of RAS Components in vitro

HUVEC were activated with TNF-α (1 ng/ml) with or without aliskiren (10⁻⁶ M) for 4 hours and then culture supernatant were collected for following EIA analysis. Renin activity in HUVEC culture supernatant were measured using a Renin activity assay kit (AnaSpec Inc., San Jose, CA) according to the manufacturers’ protocol. The value for mean fluorescence for each condition is expressed as a proportion to the control [TNF-α (-) aliskiren (-)].

The concentrations of Ang II in HUVEC culture supernatant were determined using an ELISA kit (Phoenix Pharmaceuticals) according to the manufacturer’s protocol.

Measurement of ACE Activity and Expression

ACE activity in HUVEC culture medium and lysate were evaluated by ACE activity assay kit (Life Laboratory Company, Yamagata, Japan) and WB. HUVEC were incubated with TNF-α (1 ng/ml) and with or without aliskiren (10⁻⁶ M) for 4 hours and then we collected culture medium and total cell lysate of HUVEC. We conducted ACE activity assay according to the manufacturers’ protocol. The value for mean fluorescence for each condition is expressed as a proportion to the control [TNF-α (-) aliskiren
The details about WB are described previously in this supplement method, “Western Blot Analysis” section.

Statistical Analysis

All values are expressed as the mean ± SEM. A t test and one-way analysis of variance (ANOVA), followed by Tukey post-hoc analysis were performed to analyze the differences between groups. Differences were considered to be significant when the P value was less than 0.05.

Reference for Supplemental Materials


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Supplemental Table 1. Primers for quantitative real-time PCR

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<td>mICAM-1</td>
<td>5’ - ACCCAACTGGAAGCTGTGGT - 3’</td>
<td>3’ - TGCCACAGTTCTCAAAGCAC - 5’</td>
</tr>
<tr>
<td>mVCAM-1</td>
<td>5’ - AGCCATGCATTCAGACCTGC - 3’</td>
<td>3’ - ACTTCAACGATGGGACTTG - 5’</td>
</tr>
<tr>
<td>mp22\text{phox}</td>
<td>5’ - AAGAGGGAGGGGCTGCAC - 3’</td>
<td>3’ - CGAAAGCTTTCAACACAG - 5’</td>
</tr>
<tr>
<td>mgp91\text{phox}</td>
<td>5’ - ATAGGGCTTTTCTGTTGTA - 3’</td>
<td>3’ – TGACTTCTGGGTAAAGAGC - 5’</td>
</tr>
<tr>
<td>mβ-actin</td>
<td>5’ - TTGCTGACAGGATGCAAGAGG - 3’</td>
<td>3’ - TGATCCACATCTGCTTGGAAG - 5’</td>
</tr>
</tbody>
</table>

For quantitative real-time PCR, we designed human (h) and mouse (m) specific primer pairs using primer design software Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi).
Supplemental Figure II

A  Plasma renin activity

<table>
<thead>
<tr>
<th>Injury</th>
<th>Aliskiren</th>
<th>Relative renin activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>50</td>
</tr>
</tbody>
</table>

B  Angiotensin II

<table>
<thead>
<tr>
<th>Injury</th>
<th>Aliskiren</th>
<th>Concentration (ng / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
</tbody>
</table>
Supplemental Figure III

TNF-α

Expression ratio (β-actin)

Injury
-  +  +

Aliskiren
-  -  +

P < 0.05  P < 0.05
Supplemental Figure IV

**E-selectin**

- **ICAM-1**

- **VCAM-1**

Expression ratio (β-actin)

**TNF-α**
- -
- +
- +

**Aliskiren**
- -
- -
- +

* * *

**TNF-α**
- -
- +
- +

**Aliskiren**
- -
- -
- +

* ** VCAM-1
Supplemental Figure VI

A  Renin activity

![Graph showing Renin activity with bars for different conditions: TNF-α and Aliskiren with symbols * and ** indicating statistical significance.]

B  Angiotensin II

![Graph showing Angiotensin II concentration with bars for different conditions: TNF-α and Aliskiren with symbols * and ** indicating statistical significance.]

**Note:** The graphs depict the effects of TNF-α and Aliskiren on Renin activity and Angiotensin II concentration, with statistical significance indicated by * and **.
Supplemental Figure VII

A

HUVEC Lysate

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>Aliskiren</th>
<th>Relative ACE activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>125 ± 7</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>120 ± 8</td>
</tr>
</tbody>
</table>

B

Culture Medium

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>Aliskiren</th>
<th>Relative ACE activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>125 ± 7</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>120 ± 8</td>
</tr>
</tbody>
</table>
Supplemental Figure VIII

ACE

β-actin

TNF-α  -  +  +
Aliskiren  -  -  +
Supplemental Figure IX

A

B
Supplemental Figure XI

A: Injury (-) Aliskiren (-)
B: Injury (+) Aliskiren (-)
C: Injury (+) Aliskiren (+)
Supplemental Figure XII

Ang II concentration

ng/ml

0 0.25 0.5 0.75 1

TNF-α - + + - + + - + + - + + - + +

Aliskiren - - + - - + - - + - - + - - +

hour 0.5 2 4 12

* *
Supplemental Figure XIII

AGT
Aliskiren

(RAS)

AGT
Renin

Angiotensin I
ACE
Angiotensin II

AT1-R

TNF-R

INJURY

TNF-α

Renin

(non RAS)

Aliskiren

NAD(P)H oxidase
(gp91↑)

ROS generation

MAPK (JNK ↑)

Transcription factors (NFkB ↑)

Adhesion molecules
(ICAM • VCAM • E-Selectin ↑)

Endothelial cell

Rolling & Adhesion

Vasculature
SUPPLEMENTAL FIGURE LEGENDS

Figure I. Effects of Aliskiren on Blood Pressure *in vivo*. PBS or aliskiren (3, 10, 25, 50 mg/kg/day diluted in PBS) was administered via an osmotic pump for 2 weeks. We conducted perivascular cuff placement on Day 13 and IVM analysis was performed on Day 14. We monitored blood pressure (BP) in all groups during the experimental period. SBP; systolic BP (*upper panel*), MBP; mean BP (*middle panel*), DBP; diastolic BP (*lower panel*). Values are shown as the mean +/- SEM. * p < 0.05 vs. PBS mice.

Figure II. Plasma renin activity (PRA) and Concentration of Ang II *in vivo*  
A. PRA was measured in plasma collected from mice (n=3 in each group) as described in Supplemental Methods. The value for mean fluorescence for each condition is expressed as a proportion to the control [injury (-) aliskiren (-)]  
B. Ang II concentration was evaluated (n=3 in each group) as described in Supplemental Methods. Values are shown as the mean +/- SEM.

Figure III. Expression of TNF-α *in vivo*  
The mRNA were prepared from femoral artery after perivascular cuff injury with or without aliskiren treatment as described in Methods. The mRNA expression of TNF-α was measured by quantitative real-time PCR as described in Methods. The average of three independent experiments is shown and values are shown as the mean +/- SEM.

Figure IV. Effect of Aliskiren on Expression of Adhesion Molecules in HUVEC  
HUVEC were incubated with TNF-α (1 ng/ml) with or without aliskiren (10^{-6} M) for 1 hour. Then, the mRNA expressions of E-selectin, ICAM-1, and VCAM-1 were measured by quantitative real-time PCR. The
average of three independent experiments is shown and values are shown as the mean +/- SEM. * p < 0.01 vs TNF-α (-), ** p < 0.05 vs TNF-α (+).

Figure V. Activation of NF-κB in vivo and in vitro.
Western blotting analysis was performed as described in Materials and Methods. The expressions of phosphorylated NF-κB and total IkB were compared with those of total NF-κB and β-actin proteins, respectively. Values are shown as the mean +/- SEM of three independent experiments and presented as bars below each blot. A. Lysate were prepared from HUVEC activated with TNF-α (1 ng/ml) in the presence or absence of aliskiren (10^{-7}, 10^{-6} M) for 0.5 hour. * p < 0.05 vs. TNF-α (-), ** p < 0.05 vs. TNF-α (+) aliskiren (-). B. Lysate were prepared from HUVEC activated with TNF-α (1 ng/ml) with or without aliskiren (10^{-6} M) for 2 hours, followed by additional incubation with renin (10^{-7} M) for 4 hours. * p < 0.01 vs. TNF-α (-), # p < 0.05 vs. TNF-α (+) aliskiren (-), † p < 0.01 vs. TNF-α (+) aliskiren (+). C. Lysate were prepared from mice femoral arteries after perivascular cuff injury with or without aliskiren treatment (10 mg/kg/day) for 2 weeks as described in Materials and Methods. * p < 0.05 vs. injury(-) aliskiren(-), ** p < 0.05 vs. injury (+) aliskiren (-).

Figure VI. Effect of Aliskiren on RAS components in HUVEC.
HUVEC were activated with TNF-α(1 ng/ml) with or without aliskiren (10^{-6} M) for 4 hours.

A. Renin activity was measured in culture medium as described in Supplemental Methods. The value for mean fluorescence for each condition is expressed as a proportion to the control [TNF-α(-) aliskiren (-)]

B. Ang II concentration was evaluated in culture medium as described in Supplemental Methods. Values are shown as the mean +/- SEM.

* p < 0.01 vs TNF-α (-), ** p < 0.05 vs TNF-α (+) aliskiren (-)
Figure VII. ACE activity in HUVEC

HUVEC were incubated with TNF-α (1 ng/ml) with or without aliskiren (10^{-6} M) for 4 hours and the culture medium and the cell lysate were prepared. ACE activity of HUVEC lysate (A) and their culture medium (B) was measured using ACE EIA kit (Life Laboratory Company, Yamagata, Japan) following the manufactual protocol. The average of three independent experiments is shown. The value for mean fluorescence for each condition is expressed as a proportion to the control [TNF-α(-) aliskiren (-)]. Values are shown as the mean +/- SEM.

Figure VIII. Expression of ACE in HUVEC

HUVEC were incubated with TNF-α (1 ng/ml) with or without aliskiren (10-6 M) for 4 hours and the cell lysate were prepared and ACE expression in HUVEC was evaluated by WB as described in Method.

Figure IX. Effects of exogenous renin and Ang II on TNF-α-stimulated HUVEC.

HUVEC were activated with TNF-α [1 ng/ml (A) or 0.1 ng/ml (B)] with or without aliskiren (10^{-6} M) for 2 hours, followed by additional incubation with renin (10^{-7} M) (left panel) or Ang II (10^{-7} M) (right panel) for 4 hours. Adhesion assays using monocytic THP-1 cells under flow were performed as described in Materials and Methods. Data shown are representative of three similar experiments and values are shown as the mean +/- SEM. HPF: high power field (20× microscope field). * p < 0.01 vs. TNF-α (-), # p < 0.05 vs. TNF-α (+) aliskiren (-)

Figure X. Effects of aliskiren on oxidative stress in HUVEC

HUVEC were activated by TNF-α (1 ng/ml) with or without aliskiren (10^{-6} M), followed by treatment of ROS detective probe, CM-H_{2}DCFDA as described in Materials and Methods. ROS generation in
TNF-α-stimulated HUVEC was monitored using an epi-fluorescent microscope. Representative micro-photographs (x100) showing ROS-related fluorescent intensity under the experimental conditions. NT: Non-treatment [TNF-α (-) aliskiren (-)].

Figure XI. Effect of Aliskiren on Vascular Remodeling after Cuff-injury
PBS or aliskiren (10mg/kg/day diluted in PBS) was administered via an osmotic pump for 13 days prior to perivascular cuff placement. Injured vascular segments were collected on Day 28 and fixed in 10% formaldehyde and embedded in paraffin. Serial paraffin section (3mm) were deparaffinized, dehydrated and stained with Elastica Van Gieson. Representative snapshots (x200) were demonstrated for A [injury (-) aliskiren (-)], B [injury (+) aliskiren (-)], and C [injury (+) aliskiren (+)].

Figure XII. Time Course of Ang II Concentration induced by TNF-α in Supernatant of HUVEC
HUVEC were activated with TNF-α (1 ng/ml) in the presence or absence of aliskiren (10^-6 M) for 0.5, 2, 4, and 12 hours. Ang II concentration in the supernatant was measured as described in Methods. The result was the mean value of three independent experiments and expressed as means +/- SEM. * p < 0.05

Figure XIII.
Schematic representation of the correlation between inflammation and RAS during vascular cuff-injury. The vascular injury induces pro-inflammatory cytokines including TNF-α, which promotes local renin production and initiates RAS-dependent (AT1-R mediated?) as well as -independent (RR mediated?) pathway. These stimulations induce activation of MAPK and ROS generation that lead to leukocyte-endothelial interaction. Aliskiren seems to prevent AT1-R mediated RAS signaling as well as -independent pathway in endothelial cells to exert its anti-adhesive effects. AGT: angiotensinogen. RR: renin receptor.