Oxidative Stress in Mononuclear Cells Plays a Dominant Role in Their Adhesion to Mouse Femoral Artery After Injury

Sumihiko Hagita, Mizuko Osaka, Kentaro Shimokado, Masayuki Yoshida

Abstract—Leukocyte recruitment plays a pivotal role during inflammation after vascular injury. The importance of oxidative stress in vascular injury and its modulation by angiotensin II receptor blockers (olmesartan) have been demonstrated. We examined the contribution of leukocyte-associated oxidative stress in acute-phase leukocyte recruitment and its modulation by olmesartan. Male mice were treated with olmesartan (5 mg/kg per day) or vehicle for 7 days before the transluminal wire injury of the femoral artery. Intravital microscopy of the artery revealed that the mechanical injury increased adherent leukocytes at both 24 hours and 7 days after the injury, which was significantly reduced by olmesartan treatment. Dihydroethidium-associated fluorescence intensity observed in vehicle-treated mice was significantly diminished under olmesartan treatment. Apocynin, a nicotinamide-adenine dinucleotide phosphate oxidase inhibitor, showed a similar inhibitory effect on the leukocyte adhesion. Adoptive transfer of mononuclear cells, harvested from mice after wire injury, but not from those without wire injury, exhibited adhesion to the recipient injured artery. Furthermore, olmesartan treatment of mononuclear cells, but not of injured vasculature, reduced their recruitment to the injured artery. These data indicate that leukocyte recruitment to the mechanically injured artery is mediated by oxidative stress in leukocytes but not in vasculatures. Treatment with olmesartan blocked leukocyte recruitment by antagonizing mononuclear cells-associated oxidative stress. (Hypertension. 2008;51:797-802.)

Key Words: angiotensin receptors ▪ imaging ▪ inflammation ▪ oxidant stress ▪ leukocytes

Vascular injury, including an anastomosis of coronary artery, has been shown to induce oxidative stress through activation of the renin-angiotensin system. Angiotensin (Ang) II is known to induce oxidative stress and leukocyte adhesion in the vascular wall, whereas the local renin-angiotensin system in the vasculature plays an important role in the development of injury-induced inflammation. Recent studies suggest that Ang II type 1 receptor blocker (ARB) in reducing intimal hyperplasia after mechanical injury in animal models. However, the role of ARB in the modulation of vascular inflammation at the early phase of the injury has not been fully investigated. The importance of Ang II and Ang II type 1 receptor in the development of atherosclerosis has been proposed on hypercholesterolemic mice. The anti-inflammatory effect of ARB has been demonstrated in the microvasculature of the same mouse model. Later, Petnehazy et al reported that, in the atherosclerosis model, the leukocyte-associated but not vasculature-associated Ang II signal plays a dominant role in leukocyte recruitment in the microvasculature, indicating a previously unrecognized role of Ang II-dependent oxidative stress in leukocytes during atherosclerosis. In contrast, limited information is available regarding the role of leukocytes in their recruitment to the mechanically injured artery in vivo.

Leukocyte recruitment to the site of inflamed vasculature, one of the critical mechanisms in the acute phase after injury, is a complex cascade of events in which various adhesion molecules and chemokines are involved. Recently, our group established a novel intravital microscopy (IVM) system to observe and analyze leukocyte adhesion in the mechanically injured femoral artery in the mouse. Using this novel experimental system, we were able to discover a biphasic temporal pattern of leukocyte recruitment after injury and to investigate in detail the early phase of atherosclerosis-prone vascular injury. In the present study, we examined the potential contribution of oxidative stress and its modulation by olmesartan, an ARB, in leukocyte recruitment to the mechanically injured femoral artery, as well as adoptive transfer of peripheral mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) from olmesartan-treated mice into control mice or vice versa to examine the role of specific cell populations that contribute to ARB-sensitive leukocyte adhesion.

Materials and Methods

The Methods section detailing the techniques and procedures mentioned is available in an online supplement at http://hyper.ahajournals.org.
Results

Physiological Parameters
Systolic blood pressure, heart rate, and leukocyte count (white blood cell count) during the experiment are presented in supplemental Figure S1. These parameters did not change during the drug treatment (olmesartan, 5 mg/kg per day; apocynin, 10 mg/kg per day) and wire injury of the femoral artery.

Effect of Olmesartan in Leukocyte Recruitment After Injury
Representative snapshots (Figure 1A) and video movies (Figures S2 to S5) of dynamic adhesive interaction of leukocytes to the mechanically injured femoral artery revealed that leukocyte recruitment toward the injured artery of the control animal was significantly increased 24 hours (24.25±1.59 cells per 10^4 μm² of vessel surface; n=10) and 7 days (28.38±3.38 cells per 10^4 μm² of vessel surface) after injury (open columns, Figure 1B). In contrast, olmesartan treatment significantly reduced the adhesion of leukocytes to the injured artery at 24 hours (7.42±1.02 cells per 10^4 μm² of vessel surface) and 7 days (13.00±0.83 cells per 10^4 μm² of vessel surface) after injury (solid columns, Figure 1B). The rolling velocity of leukocytes, an important parameter for rolling interaction, significantly increased in the olmesartan treatment group as compared with the control group (Figure 1C), although the rolling influx was not significantly changed between these 2 groups (data not shown). Olmesartan inhibited the intimal hyperplasia of the murine femoral artery at 28 days after mechanical injury (Figure S6), as reported previously.8

Role of Oxidative Stress in Leukocyte Recruitment After Vascular Injury
Because oxidative stress has been shown to play a pivotal role in vascular injury, we estimated its level by infusing dihydroethidium (DHE), a fluorescence probe used to detect an oxidative stress. As shown in Figures 2A and 2B, vascular injury significantly increased DHE-sensitive oxidative stress, which was blunted by olmesartan treatment at 24 hours, as well as 7 days after injury. To confirm the contribution of a reduction of oxidative stress in antiadhesive property of olmesartan, we examined the effect of apocynin, a free radical scavenger. As clearly shown in snapshot pictures (Figure 3A) and their quantifications (Figure 3B), the number of leukocytes adhered to the vascular wall was significantly lower in the apocynin-treated group (apcy) as compared with the group treated with vehicle at 24 hours (group treated with vehicle, 20.45±3.66 cells per 10^4 μm² of vessel surface; apcy, 10.22±0.95 cells per 10^4 μm² of vessel surface; n=10; P<0.05) and 7 days (group treated with vehicle, 22.21±4.45 cells per 10^4 μm² of vessel surface; apcy, 13.83±1.56 cells per 10^4 μm² of vessel surface; n=6). Rolling velocity was also similarly increased to that in the olmesartan group (Figure 3C). As expected, apocynin inhibited injury-induced oxidative stress in vasculature both at 24 hours and 7 days after injury (Figure 3D).
Olmesartan Treatment on Leukocytes, but Not of the Vasculature, Inhibits Leukocyte Recruitment to the Injured Femoral Artery

To determine whether olmesartan affects the leukocytes or the injured vascular tissues, we performed adoptive transfer of peripheral MNCs. First, MNCs from nontreated mice were harvested 24 hours after wire injury, labeled ex vivo with Rhodamine 6G, and administered intravenously into nontreated recipient mice with wire injury. As shown in Figure 4A, this led to significant leukocyte recruitment in the recipient femoral artery. In contrast, when MNCs, prepared from nontreated mice without wire injury, were infused into nontreated recipient mice with wire injury, MNC recruitment was significantly reduced even though the recipient artery was injured comparably (Figure 4B). This finding reveals a dominant role of MNCs, but not vasculature, in mediating MNC recruitment to the mechanically injured artery. Next, we examined a potential effect of olmesartan in MNCs and in the injured vasculature. When MNCs harvested from mice treated with olmesartan were infused into recipient mice, MNC recruitment in the recipient injured artery was significantly inhibited (Figure 4C). In contrast, when MNCs prepared from mice without olmesartan were injected into recipient mice with olmesartan, significant MNC recruitment was observed (Figure 4D). Quantitative analyses confirmed our findings (Figure 4E). Similar inhibitory effects for MNCs were also confirmed with apocynin (Figure 4E).
them to those without olmesartan treatment at 24 hours and 7 days after injury (Figure 5) and compared them with the effects for MNCs. MNCs exhibited similar levels of adhesion to the injured vasculature at both 24 hours and 7 days after injury, which was significantly reduced when MNCs were prepared from those treated with olmesartan. In contrast, adhesion of PMN was prominent at 24 hours but not 7 days after injury. Olmesartan treatment significantly reduced PMN adhesion at 24 hours after injury (Figure 5).

Oxidative Stress and Integrin Expression in Circulating Leukocytes

To detect the increase of oxidative stress in MNCs in the injured mice, we performed flow cytometry for DHE. As shown in Figure 6A, DHE-associated fluorescence intensity in MNCs was increased after mechanical injury and reduced after olmesartan treatment. Furthermore, we analyzed the expression levels of cell surface CD11b, a member of the integrin family of adhesion molecules. As shown in Figure 6B, the expression level of CD11b was increased after mechanical injury and reduced after olmesartan treatment. Apocynin-treated MNCs also exhibited reduction of DHE staining, as well as CD11b expression.

Discussion

This study demonstrates that ARB (olmesartan) inhibits leukocyte recruitment to the vascular injury via modulation of oxidative stress in leukocytes without affecting systemic physiological parameters. Although olmesartan has been demonstrated to reduce intimal hyperplasia at 14 or 28 days after injury, its effect in the early phase after injury has not been examined. As we demonstrated recently, the mechanical vascular injury induces leukocyte adhesion at 4 hours, 24 hours, and 7 days after injury. Olmesartan significantly reduced the number of adherent cells and increased rolling velocity at both 24 hours and 7 days after injury. Although Ang II is considered as one of the most potent inflammation mediators in vivo, its effect in leukocyte recruitment to the injured vasculature has not been demonstrated, except for relatively small venules or arterioles. In this article, however, we have been able to demonstrate that the blockade of an Ang II–dependent pathway by ARB has an antiadhesive effect in the mechanical injury of a mouse femoral artery. Our data suggest that an anti-inflammatory effect of ARB can be observed as early as 24 hours after injury. Previous reports described that olmesartan has the antioxidative property of inhibiting nicotinamide-adenine dinucleotide phosphate oxidase activity in the injured arteries of high-fat diet–treated apolipoprotein E knockout mice. In other reports, superoxide anion or reactive oxygen species have been reported to induce leukocyte and endothelial activation. Therefore, we examined the potential contribution of oxidative stress in leukocyte recruitment observed in our injury model. In fact, apocynin, a nicotinamide-adenine dinucleotide phosphate oxidase inhibitor, reduced leukocyte recruitment at both 24 hours and 7 days after injury. Furthermore, olmesartan, as well as apocynin, has been shown to reduce oxidative stress formation judging from DHE fluorescence, which confirmed the importance of oxidative stress in the process.
We further tried to define the specific cell populations responsible for the mechanical injury–induced leukocyte recruitment and its blockade by olmesartan. Despite its importance in the vascular wall, the oxidative stress in circulating MNCs had not been carefully studied until recent observation of the microvasculature of hypercholesterolemic mice, in which the importance of the Ang II type 1 receptor on leukocytes was addressed for the first time. Indeed, although MNCs from injured mice exhibited significant adhesion to the recipient injured artery, MNCs taken from mice without wire injury failed to adhere to the injured artery (Figures 4B and 4C), suggesting a dominant role of MNCs in leukocyte recruitment to the femoral artery after injury. We also observed a significant adhesion of PMN at 24 hours but not 7 days after injury, suggesting a contribution of PMNs in the acute phase of injury, although their number is relatively small (~15% of peripheral leukocytes) in mice. The temporal pattern of adhesive interaction of PMNs was parallel to the reduction of oxidative stress at the vasculature (Figure 5), suggesting their role as a source of oxidative stress at the acute phase of vascular injury.

Although our model demonstrated a pattern of leukocyte recruitment after vascular injury, there is a substantial difference between leukocyte recruitment observed in hypercholesterolemic mice where leukocytes interact with endothelial cells and that observed in mechanically injured mice where leukocytes interact with the denuded vascular wall. Nonetheless, our observations, in which circulating MNCs play an important role, represent a novel insight in the pathophysiology of vascular injury, such as percutaneous coronary intervention. Similarly, the antiadhesive effect of olmesartan was prominent in MNCs when compared with its effect on vascular cells. In fact, oxidative stress in MNCs was significantly elevated after wire injury and inhibited when olmesartan was administered (Figure 6A). Although the precise molecular mechanisms responsible for the generation of oxidative stress in MNCs were not known, the moderate upregulation of CD11b (Figure 6B) supported our adhesion data. As reported previously, oxidative stress has been shown to enhance CD11b expression in THP-1 cells treated with nonesterified fatty acids, primarily through the nicotinamide-adenine dinucleotide phosphate oxidase–dependent pathway. Because we observed cell adhesion after intimal denudation, we should examine other atherosclerosis models without luminal injury to monitor leukocyte adhesion to the vascular wall in the presence of an endothelial layer to understand leukocyte adhesion during the atherogenesis process.

**Perspectives**

We observed that olmesartan, an ARB, inhibits leukocyte recruitment as early as 24 hours after the mechanical injury of the femoral artery via the modulation of oxidative stress in MNCs. Our findings provide a novel insight to postulate a broader role for oxidative stress in mediating adhesive interaction to vascular injuries and a potential application of olmesartan as a treatment in the early phase of vascular injury to prevent inflammatory responses.

**Acknowledgment**

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**Disclosures**

None.

**References**


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Oxidative stress in mononuclear plays a dominant role in their adhesion to mouse femoral artery after injury

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Running title: oxidative stress induces monocyte adhesion

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Online Materials and Methods

Reagents

Olmesartan Medoxomil (RNH-6270) was kindly provided by Daiichi Sankyo Co., Ltd., Tokyo, Japan. Apocynin was purchased from Calbiochem (San Diego, CA, USA).

Animals

Male C57BL/6J mice (7 w of age) were obtained from Oriental Yeast (Tokyo, Japan). Animals were fed with standard chow (Clea Japan, Inc., Japan); food and water were provided ad libitum. The experiments were approved by the ethical committee for animal experimentation of Tokyo Medical and Dental University, Tokyo. Olmesartan Medoxomil (5 mg /kg/day), apocynin (10 mg/kg/day), or vehicle (Sodium Bicarbonate for olmesartan and distilled water (D.W.) for apocynin) were administered orally for 7 days before wire injury and continued until IVM analysis. Systolic blood pressure and heart rate during experiments were measured through tail cuff with noninvasive automatic sphygmomanometer (BP-98A, Softron, Tokyo, Japan).

Mechanical injury of femoral artery was induced by insertion of a large wire
(0.38mm in diameter, No. C-SF-1515, Cook, Bloomington, IN) as previously described. In brief, after mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Nembutal, Abbott Laboratories, Abbott Park, Ill) femoral artery was exposed and clamped the upstream and the downstream of arterial blanch. Then the guide wire was inserted to femoral artery and removed from vessel followed by the branch ligation. This procedure caused complete endothelial denudation. The operative time for femoral injury ranged from 20 to 30 minutes and all mice survived until the time of microscopic examination.

Intravital microscopy (IVM)

Intravital microscopic examination of the injured femoral arteries was carried out at 24 hours and 7 days after wire injury as previously described. In brief, mice were anesthetized with pentobarbital as described above and mechanically ventilated so as to maintain normal acid-base balance. Rectal temperature was kept at 36.0-37.0 °C with a heating pad and an infrared heat lamp. After injection of rhodamine 6G chloride (Molecular Probe; 0.3 mg/kg in 300 μl of Phosphate buffered saline (-)) to the right femoral vein, the injured femoral artery was visualized with a fluorescent microscope (BX51WI, Olympus, Tokyo) equipped
with a water immersion objective (x20). Epifluorescence was illuminated by a 100W fluorescent lamp source and images were directly captured to a PC via a CCD camera (CoolSnap HQ, Olympus, Tokyo, Japan). Each experimental group consisted of at least 10 mice (Olmesartan group and control group) or 5 mice (Apocynin group and D.W. group).

Image analysis

Adhesion of leukocytes was clearly visualized on the anterior half of the vessels facing the objective. All images were analyzed using an image analysis program (Meta morph) in accordance with the manufacturer’s protocol as previously described. In brief, the number of adherent cells during the one-minute recording period was counted along a region of interest (ROI), a 100 μm by 100 μm rectangle segment of the vessel and expressed as the number of adherent cells/10^4 μm^2 of the vessel surface. The frequency of rolling interactions was determined by counting fluorescent cells that moved, passing a reference line perpendicular to the vessel axis. The results were expressed as the number of rolling cells/minutes/10^3 μm of the vessel perimeter, in order to adjust the differences in vessel size. We calculated the mean rolling velocity of rolling
leukocytes from the total distance measured by tracking individual rolling leukocytes over a distance of 10 to 50 mm, and their elapsed time.

Detection of local oxidative stress

To detect oxidative stress in the injured femoral artery in vivo, we conducted IVM after intravenously injection of dihydroethidium (DHE), a fluorescent dye used to quantify oxidative stress production. IVM was performed 5 minutes after injection of DHE and the DHE-associated fluorescence of cells and injured vasculature was observed under a fluorescent microscope. The magnitude of oxidative stress was estimated as a ratio of the fluorescence positive areas to the captured images.

Adoptive transfer of leukocytes

MNCs of the peripheral blood were isolated from 3 mice with or without wire injury by gradient centrifugation using lymphocytic separation medium (Histopaque -1083, Sigma, USA). The cells (1×10^6 cells per mouse) were labeled with rhodamine 6G chloride and injected intravenously into the other mice for IVM observation. In some experiments, mice were treated with
olmesartan or apocynin before wire injury or MNCs preparation. In other experiments, polymorphnuclear neutrophils (PMN) prepared by a magnetic cell sorting method using Anti-Ly-6G MicroBead Kit (Miltenyi Biotec K.K., Germany) were labeled with rhodamine 6G chloride and injected intravenously into the other mice for IVM observation.

Flow cytometry

MNCs prepared from 3 mice of each group (at 24 hr after injury) were suspended in RPMI1640 / 5% fetal bovine serum. For the detection of intracellular oxidative stress, the cells were incubated in the presence of DHE (1:250) for 20 min at 37°C. For the detection of cell surface CD11b, the cells were first incubated with an anti-mouse CD11b (1:10, MCA74, Serotec, USA) for 45 min at 22°C followed by fluorescein isothiocyanate isomer (FITC) -conjugated secondary antibody (1:20, R&D Systems, Inc., USA) for 45 min at 22°C. After 3 washings, the fluorescence activity was detected from 10 000 MNCs fraction using FACS caliber at 580 nm (DHE) or 480 nm (CD11b), and the data was analyzed by CellQuest software (Becton Dickinson).
Statistical analysis

Data are expressed as mean value ± SEM. One-way ANOVA with Tukey post-hoc test or two-tailed unpaired $t$ test was used to estimate statistical significance. A value of $p<0.05$ was considered as statistically significant.

Online Figure Legend

Figure S1
Heart rate, Systolic blood pressure, and WBC counts of C57BL/6J mice during experiments.
Heart rate (HR) (A), Systolic blood pressure (SBP) (B), and the number of leukocytes (WBC) (C) at the time point where oral administration (-7), before wire injury (0), 24 hour after wire injury (+1) and 1 week after wire injury (+7). Values are the mean ± SEM of at least 10 (olmesartan and NC) or 6 (apocynin) mice at each group.

Figure S2-S5
Representative movies of femoral artery of mice. S2 and S3 are movies of vehicle treatment group at 24hr or 7 days after wire injury, respectively and S4 and S5 are movies of mouse treated olmesartan.

Figure S6
Inhibitory effect of olmesartan for intimal hyperplasia after mechanical injury. HE stain of femoral artery section of mice at 28 days after wire injury. Olmesartan treatment group (A) significantly reduced intimal hyperplasia after mechanical injury compared to vehicle treatment group (B).
Figure S1

A

HR (m/min)

0 200 400 600 800

-7 0 +1 +7

B

SBP (mmHg)

80 90 100 110 120 130 140

-7 0 +1 +7

C

WBC counts

(\times 10^6 \text{ cells / ml blood})

0 2 4 6 8

-7 0 +1 +7

- Vehicle
- ARB
- Apocynin
Figure S2 ~ S5

<These figures are AVI movie files and separately uploaded>
Figure S6