Leukocyte-Dependent Responses of the Microvasculature to Chronic Angiotensin II Exposure

Alper Yildirim, Janice Russell, Li-Sue S. Yan, Elena Y. Senchenkova, D. Neil Granger

Abstract—Angiotensin II (Ang II) contributes to the pathogenesis of hypertension and other cardiovascular diseases. Ang II induces a pro-oxidative, proinflammatory, and prothrombogenic phenotype in vascular endothelial cells. Although the peptide promotes the recruitment of leukocytes and platelets and induces oxidative stress in the microvasculature, it remains unclear whether and how the blood cell recruitment is linked to the production of reactive oxygen species. In this study, we addressed the contributions of Ang II type 1 receptors (AT₁r) and gp91phox to the recruitment of leukocytes and platelets and reactive oxygen species production in venules during chronic (2-week) infusion of Ang II in wild-type (WT) and mutant mice. Intravital video microscopy was used to measure the adhesion and emigration of leukocytes, the adhesion of fluorescently labeled platelets, and dihydrodorhamine oxidation (a measure of oxidative stress) in cremaster muscle postcapillary venules. In WT mice, Ang II infusion induced a time-dependent increase in the adhesion of leukocytes and platelets and enhanced reactive oxygen species production in venules. These changes in blood cell adhesion and reactive oxygen species production were not observed in AT₁r−/− mice, AT₁r−/− bone marrow chimeras (blood cells deficient in AT₁r), gp91phox−/− mice, gp91phox−/− chimeras (blood cells or endothelial cells deficient in gp91phox), and in WT mice rendered granulocytopenic via intraperitoneal injection of antimouse granulocyte receptor 1 antibody. Thrombocytopenic WT mice (platelets depleted by intraperitoneal injection of rabbit antimouse thrombocyte antiserum) responded similar to WT mice. These findings implicate leukocyte-associated AT₁r and gp91phox in the induction of the pro-oxidative, proinflammatory, and prothrombogenic phenotype assumed by microvessels that is chronically exposed to elevated Ang II. (Hypertension. 2012;60:00-00.) ● Online Data Supplement

Key Words: angiotensin II ▪ neutrophils ▪ oxidative stress ▪ blood vessels ▪ microcirculation

Hypertension (HTN) is one of several risk factors for cardiovascular disease that can lead to structural and functional alterations in both large and microscopic blood vessels. It is now widely appreciated that the blood vessel responses to HTN result from physical and chemical factors that exert an influence on the endothelial and smooth muscle components of the vessel wall. Angiotensin II (Ang II), a product of the renin-angiotensin system, has received considerable attention as a circulating soluble factor that is capable of mediating the elevated blood pressure, as well as other phenotypic changes, in blood vessels that accompany HTN and other risk factors (eg, hypercholesterolemia). Ang II is a pleiotropic agent that has been implicated as a mediator of the oxidative stress, immune cell activation and recruitment, thrombogenesis, and the impaired vasomotor and endothelial barrier functions caused by cardiovascular disease risk factors. The profile of vascular changes that are induced by Ang II suggests that this vasoactive peptide may play an equally important role as an inflammatory mediator.

An important feature of the proinflammatory actions of Ang II in the microvasculature is its ability to activate a variety of cells that normally circulate in blood (leukocytes and platelets) or that reside within the vessel wall (endothelial cells). Most of the evidence in the literature indicates that Ang II mediates the activation of these different cell populations by engaging with angiotensin II type 1 receptors (AT₁r). An accelerated production of reactive oxygen species (ROS) is observed in microscopic vessels that are either acutely or chronically exposed to Ang II. The vascular wall oxidative stress induced by Ang II has been linked to AT₁r and it is generally attributed to reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase–dependent ROS production by endothelial cells. However, both leukocytes and platelets, which are recruited onto the vessel wall in response to Ang II, are known to express AT₁r and NADPH oxidase and can increase ROS production in response to Ang II. This raises the possibility that some of the ROS production that is detected in microvessels exposed to elevated Ang II may result from the activation of adherent leukocytes and platelets, perhaps via the engagement of AT₁r on the circulating cells. This possibility appears tenable in view of previous reports that describe a major contribution of adherent leukocytes to...
the oxidative stress associated with other models of microvascular inflammation.\textsuperscript{14-16}

The overall objective of this study was to determine whether the recruitment of adherent neutrophils and platelets in venules during chronic Ang II exposure contributes to the oxidative stress that accompanies this condition. In addition, using bone marrow chimeras, we assessed the relative contributions of blood cell- versus endothelial cell-associated NADPH oxidase (gp91phox) and AT\textsubscript{1}r to the oxidative stress and inflammatory cell recruitment elicited by Ang II. Our findings indicate that neutrophil-associated NADPH oxidase plays a major role in Ang II-mediated oxidative stress and implicates blood cell--associated AT\textsubscript{1}r in the increased ROS production.

**Methods**

All of the mice used in the experimental studies were on a C57BL/6 background, and the mutant mice were backcrossed to C57BL/6 for ≥7 generations. Male wild-type (WT) C57BL/6J, AT\textsuperscript{1}r\textsuperscript{−/−}, and gp91\textsuperscript{1−/−} (B6.129S6-Cybph{	extsuperscript{−/−}}/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME) or derived from an established breeding colony (eg, AT\textsuperscript{1}r\textsuperscript{−/−}) on our campus. All of the experimental procedures involving the use of animals were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

**Bone Marrow Chimaera Production**

Bone marrow transfer was used to create chimeric mice (denoted as donor→recipient), that is, WT→WT, gp91\textsuperscript{1−/−}→WT, AT\textsuperscript{1}r\textsuperscript{−/−}→WT, and WT→gp91\textsuperscript{1−/−}→WT, as described previously.\textsuperscript{6,9} For a more detailed method, see the online-only Data Supplement.

**Osmotic Pump Implantation**

Saline or Ang II (1 \textmu g/kg per minute) was infused over 3, 7, 10, or 14 days using micro-osmotic pumps (model 1002, ALZET, Cupertino, CA), which were implanted subcutaneously in the intrascapular region of isoflurane-anesthetized mice, as described previously.\textsuperscript{5} Blood pressure was measured by tail-cuff plethysmography (model SC-1000, Hatteras Instruments, Inc, Cary, NC) in nonanesthetized animals.

**Surgical Protocol**

Mice were anesthetized with ketamine hydrochloride (150 mg/kg of body weight intraperitoneally) and xylazine (7.5 mg/kg of body weight intraperitoneally). The right jugular vein was cannulated for administration of heparinized saline and platelets, and the right carotid artery was cannulated for systemic arterial pressure measurement. The cremaster muscle was prepared for intravital fluorescence microscopic observation and the adhesion of leukocytes and platelets in cremaster muscle venules was determined, as described previously.\textsuperscript{17,18} A more detailed description of the intravital microscopic methods used to evaluate blood cell-endothelial cell interactions in cremaster venules is provided in the online-only Data Supplement.

**Platelet Preparation**

Male WT mice were used as platelet donors. Platelets were isolated from whole blood by a series of centrifugation steps, labeled with the fluorochrome carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR), as described previously,\textsuperscript{7} and resuspended in phosphate-buffered saline at a concentration of 8.33 × 10\textsuperscript{8} cells/\textmu L. This technique does not cause platelet activation as determined by P-selectin expression using flow cytometry.\textsuperscript{18}

**Dihydrorhodamine Oxidation**

The production of ROS in cremaster venules was determined by monitoring the oxidation of dihydorhodamine (DHR), as described previously.\textsuperscript{19} DHR oxidation was monitored in cremaster preparations separate from those used to monitor blood cell adhesion. Background fluorescence (I\textsubscript{0}) of the first 100 \textmu m of every 300-\textmu m vessel length was recorded along the length of selected postcapillary venules with a xenon light source and an FITC camera system (Hamamatsu). Then, freshly prepared dihydorhodamine-123 (1 mmol/L, a nonfluorescent dye that is oxidized by ROS to the fluorescent compound rhodamine-123) in bicarbonate-buffered saline was superfused over the cremaster for 15 minutes. The tissue was then washed with bicarbonate-buffered saline and the fluorescent image of each section recorded (I\textsubscript{final}). Images were captured onto a computer, and an area 100 \textmu m long and 7 \textmu m wide along the vessel wall was analyzed in each vascular segment using National Institutes of Health Image 1.62 software. The ratio of I\textsubscript{final}/I\textsubscript{0} was calculated for each vascular segment, and the average ratio for each animal was determined.

**Blood Cell Analyses**

Whole blood samples (20–25 \textmu L) were obtained from each mouse for determination of leukocyte (stained with 3% acetic acid and 10% crystal violet) and platelet (stained with 1% buffered ammonium oxalate) counts using a hemocytometer. The flow-cytometric methods used to identify and quantify the different circulating leukocyte populations in control and Ang II–infused mice are summarized in the online-only Data Supplement.

**Experimental Protocols**

An initial study was directed to defining the time course of changes in the adhesion of leukocytes and platelets, leukocyte emigration, and DHR oxidation (oxidative stress) in cremaster muscle venules and blood pressure. In 5 groups of mice (n=25), all of these variables were measured on days 0, 3, 7, 10, and 14 using the intravital video microscopic procedures as described above. All of the subsequent experiments were performed in mice exposed to Ang II infusion for 14 days. Some WT mice with Ang II pumps were rendered thrombocytopenic via an intraperitoneal injection of antplatelet serum (125 \textmu L/kg in 200 \textmu L of phosphate-buffered saline intraperitoneally; rabbit antimouse thrombocyte antiserum, Accurate Chemicals, Westbury, NY) 24 hours before intravital microscopy (n=5). Treatment with antiplatelet serum reduced the number of circulating platelets by ≥91%, without significantly reducing the number of circulating leukocytes, as confirmed by measurements of blood cells with a hemocytometer. The effects of neutrophil depletion on Ang II–induced microvascular responses were examined in mice using 2 different antibodies, that is, antigranulocyte receptor-1 RB6-8C5 (eBioscience, 100 \textmu L per mouse) or the antimouse Ly-6G antibody 1A8 (BioLegend, 150 \textmu L per mouse). Both antibodies were delivered by intraperitoneal injection 24 hours before intravital microscopy (n=7 and n=5, respectively). Treatment with RB6-8C5 reduced the number of circulating neutrophils by >90% while showing no effect on the number of the circulating platelets, whereas 1A8 treatment reduced number of neutrophils by ≤71%.

**Spinning Disk Confocal Microscopy**

In a separate group of mice, an effort was made to define the different leukocyte populations that adhered in cremaster muscle venules on day 14 of Ang II infusion. A spinning disk confocal microscope was used to visualize different leukocyte subpopulations that were labeled with fluorescently labeled antibodies that target specific surface proteins expressed by the different cell populations. A more detailed description of the methods used for this analysis is provided in the online-only Data Supplement.

**Statistics**

Data were analyzed using standard statistical analysis, that is, 1-way ANOVA and Tukey post hoc test and Fisher least significant difference test, as appropriate. A Student t test was used to compare differences between 2 groups. All of the values are reported as mean±SEM from 5 to 14 mice, and statistical significance was set at P<0.05.
Results

Ang II pump implantation yielded a significantly elevated blood pressure in WT mice (132.6±2.8 mmHg) compared with WT mice with a saline-loaded pump and WT mice not subjected to pump implantation (99.4±0.5 mmHg). Shear rates did not differ significantly between the experimental groups for both arterioles and venules (data not shown). The Ang II–induced HTN was not observed in AT1r−/− mice. Table 1 summarizes the effects of Ang II on systolic blood pressure in the different experimental groups. The time course studies revealed that blood pressure was significantly elevated beginning on day 3 of Ang II infusion. All of the mice except AT1r−/− with an implanted Ang II loaded pump exhibited a significantly elevated systolic blood pressure compared with WT mice. AT1r−/− chimeras showed no attenuation of blood pressure compared with WT mice.

Figure 1 demonstrates that mice with an Ang II pump exhibit a significant increase in the number of leukocytes adhering in postcapillary venules on days 3, 7, 10, and 14 of pump implantation when compared with control mice (Figure 1A). Leukocyte emigration was also increased in the Ang II–infused mice on day 14 versus control mice (Figure 1B). The Ang II–induced adhesion of leukocytes was accompanied by substantial platelet recruitment. Although platelet adhesion was elevated at all of the time points, only on days 10 and 14 were the increases statistically significant when compared with control animals (Figure 1C). The DHR oxidation measurements revealed that oxidative stress in venules is evident (statistically significant) on day 7 of Ang II pump infusion, and it remains elevated for the remainder of the infusion period (Figure 1D). Figure S1 shows representative images of dihydrorhodamine oxidation within and surrounding cremaster muscle venules in the different experimental groups on day 14 of Ang II infusion.

Figure 2 illustrates the contribution of blood cell–associated and endothelial cell–associated AT1r to the microvascular responses elicited by Ang II infusion. The numbers of adherent leukocytes in postcapillary venules of WT+Ang II mice were significantly higher than those observed in control mice (Figure 2A). The Ang II–induced increase in adherent leukocytes was significantly blunted in AT1r−/− mice and in AT1r−/−→WT chimeras (Figure 2A). Leukocyte emigration was also increased in the Ang II+WT mice compared with the control group (Figure 2B). Much like the leukocyte adherence

Table. Ang II–Mediated Changes in Systolic Blood Pressure in WT (Control), AT1r−/−, Chimeric (AT1rCh), Neutrophil-Depleted, Platelet-Depleted, gp91phox−/−, and gp91phox−/−Ch

<table>
<thead>
<tr>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Ang II</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Before pump implantation</td>
<td>99.9±0.6</td>
</tr>
<tr>
<td>Day 14 of pump implantation</td>
<td>132.6±2.8*</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II; WT, wild-type; AT1r−/−, Ang II type 1 receptor knockout; AT1rCh, AT1r−/− bone marrow chimeras. *P≤0.05 vs control (WT–before pump implantation).
responses, a profound reduction in leukocyte emigration was noted in both AT1r−/− and AT1r−/−→WT mice. The number of adherent platelets in the WT+Ang II mice was increased compared with control mice. This response to Ang II was completely abolished in AT1r−/− mice and AT1r−/−→WT mice (Figure 2C). Ang II administration also increases DHR oxidation levels (Figure 2D and Figure S1B) compared with control mice (Figure S1A); however, this effect was significantly attenuated in both AT1r−/− mice (Figure S1C and Figure 2D) and AT1r−/−→WT mice (Figure 2D).

Figure 3 demonstrates the effects of short-term granulocyte (with the antigranulocyte receptor 1 antibody, RB6-8C5) or platelet depletion on Ang II–induced blood cell recruitment. In mice rendered granulocytopenic (neutrophil-depleted group), leukocyte adhesion (Figure 3A) and emigration (Figure 3B) were significantly reduced to control levels. Treatment with

Figure 2. Angiotensin II (Ang II)–mediated changes in leukocyte adhesion (A), emigration (B), platelet adhesion (C), and dihydrorhodamine (DHR) oxidation (D) in wild-type (WT) and Ang II type 1 receptor-deficient (AT1r−/−) mice and in AT1r−/−→WT mice. *P<0.05 relative to WT control; #P<0.05 relative to Ang II-WT.

Figure 3. Angiotensin II (Ang II)–mediated changes in leukocyte adhesion (A), leukocyte emigration (B), platelet adhesion (C), and dihydrorhodamine (DHR) oxidation (D) in untreated (control) wild-type (WT) mice and WT mice treated with either antigranulocyte serum or antplatelet serum. *P<0.05 relative to WT control; #P<0.05 relative to WT+Ang II.
another antigranulocyte antibody (1A8) exerted the same level of protection for all of the microvascular responses (data not shown), as noted in Figure 3 for RB6-8C5 treatment. In platelet-depleted animals, leukocyte adhesion (Figure 3A) was only partially reduced and emigration (Figure 3B) was not altered compared with control animals. Ang II–induced platelet recruitment into venules of granulocytopenic mice was reduced to control values (Figure 3C). Rendering the mice granulocytopenic largely prevented the oxidative stress induced by chronic Ang II infusion (Figures S1D and 3D). However, in thrombocytopenic animals (Figure S1E), DHR oxidation did not differ from the value detected in WT+Ang II mice (Figures S1E and 3D).

Figure S2 summarizes the confocal microscopic analysis of leukocyte subpopulations that are recruited into venules in response to Ang II infusion. As noted with light microscopy, Ang II infusion is associated with a significant recruitment of CD45+ (total) leukocytes. Although granulocytes appear to represent the largest population of recruited leukocytes, monocytes, T lymphocytes, and B lymphocytes also appear to accumulate in the vessels as a result of Ang II stimulation. The leukocyte counts in blood for the different subpopulations were not altered by Ang II, except for T lymphocytes, which exhibited a statistically significant increase (data not shown). An analysis of the formation of platelet aggregates with different leukocyte populations in Ang II–infused mice revealed a significantly increased formation of platelet-neutrophil aggregates, without any change in aggregate formation of platelets with either monocytes or lymphocytes (Figure S3).

Figure 4 summarizes the results of experiments designed to address the role of NADPH oxidase in mediating the oxidative stress and increased blood cell recruitment elicited by chronic Ang II infusion. Ang II–induced leukocyte adhesion was significantly attenuated in both gp91phox−/− and gp91phox−/−→WT mice (Figure 4A). The number of emigrated leukocytes was also reduced in both groups; the reduction achieved statistical significance in gp91phox−/− and gp91phox−/−→WT mice (Figure 4B). Ang II–induced platelet adhesion was not altered in either gp91phox−/− or gp91phox−/−→WT mice (Figure 4C). However, the oxidative stress elicited by Ang II was largely prevented by gp91phox deficiency in either all of the cells (gp91phox−/− mice; Figures S1F and 4D) or only in circulating blood cells (gp91phox−/−→WT mice; Figure 4D). Experiments with WT→gp91phox−/− chimeras (NADPH oxidase deficiency in the vessel wall but not in blood cells) revealed significant reductions in leukocyte adhesion and emigration and DHR oxidation that were comparable to those observed in gp91phox−/−→WT chimeras.

**Discussion**

The link between oxidative stress and Ang II–mediated vascular dysfunction is well established. Ang II–mediated ROS production has been proposed to be a critical event in the induction of an inflammatory phenotype in the vasculature caused by cardiovascular disease risk factors, including HTN and hypercholesterolemia. The prevailing view of Ang II–mediated inflammation is largely based on the assumption that endothelial cells are the principal target of Ang II action and that the engagement of Ang II with AT1r on endothelial cells leads to an accelerated production of ROS via NADPH oxidase. The resultant oxidative stress enhances the generation of inflammatory mediators, increases adhesion molecule expression and endothelial adhesivity, and promotes the subsequent recruitment and activation of adherent leukocytes and platelets on the endothelial cell surface. Although much attention has been devoted to the role of endothelial cells in the vascular oxidative stress induced by Ang II, the role of blood cells in this process is not well understood.
II, less is known about the contribution of recruited/adherent blood cells to the accelerated ROS production elicited by Ang II. The results of the present study suggest that neutrophils, but not platelets, play a major role in eliciting the venular wall oxidative stress that accompanies chronically elevated Ang II levels and that the neutrophil-dependent oxidative stress is mediated via an NADPH oxidase–dependent mechanism.

The findings of this study provide several lines of evidence that suggest a role for blood cells in the oxidative stress elicited in venules by chronic Ang II infusion: (1) the time course of blood cell recruitment into postcapillary venules during the 14 days of Ang II infusion parallels the increased intensity of the oxidative stress exhibited in these vessels (Figure 1); (2) bone marrow chimeras that are deficient in blood cell–associated AT1Rs or NADPH oxidase exhibit blunted oxidative stress responses to chronic Ang II exposure that are comparable with the response observed in the respective knockout (AT1R−/− or gp91phox−/−) mice (Figures 2 and 4); and (3) selective depletion of circulating neutrophils (but not platelets) effectively prevents Ang II–mediated oxidative stress (Figure 3). Collectively, these lines of evidence are consistent with a mechanism whereby the elevated circulating Ang II levels engage with AT1R on neutrophils to activate NADPH oxidase. The oxidative stress detected in venules may either reflect attached activated neutrophils that produce ROS or an activating effect of neutrophils on endothelial cells, which, in turn, produce ROS at an accelerated rate. The latter possibility is supported by our observation that gp91phox−/−→WT and WT→gp91phox−/− bone marrow chimeras produced the same level of attenuation of oxidative stress, suggesting that the activation of NADPH oxidase in one cell population (e.g., endothelial cells) is required for enzyme activation (and oxidative stress) in the other cell type (e.g., leukocytes). Such a series-coupled relationship between endothelial cell and leukocyte NADPH oxidases is consistent with the results of a previous report wherein gp91phox−/−→WT and WT→gp91phox−/− bone marrow chimeras exhibited a similar level of attenuation of leukocyte and platelet adhesion in cremaster muscle venules of hypercholesterolemic mice.22 This shared characteristic of the Ang II infusion and diet-induced hypercholesterolemia may reflect the dependence of oxidative stress induction in both models on AT1Rs.25

The importance of neutrophils in Ang II–mediated microvascular alterations is supported by several reports in the literature.4,9,10 Acute Ang II exposure has been shown to induce the adhesion of leukocytes to monolayers of cultured endothelial cells26 and in postcapillary venules,5,10 with neutrophils accounting for most of the adherent leukocytes in vivo. This is consistent with our observation that most of the leukocyte adhesion elicited by chronic Ang II exposure is abolished by rendering mice neutropenic. The dominant role of neutrophils in this model is also evident from our confocal imaging studies, which reveal that neutrophils account for a large majority of adherent leukocytes that adhere in Ang II–stimulated venules (Figure 3). Equally important is our observation that other leukocyte populations, including T cells and B cells, adhere in venules in response to Ang II, albeit to a more limited extent than neutrophils. The pathophysiological importance of these smaller populations of recruited leukocytes to the oxidative stress and vascular dysfunction elicited by Ang II remains unclear.

Our findings in neutropenic mice also suggest that neutrophil recruitment is an important determinant of Ang II–mediated platelet recruitment. Studies in other experimental models of chronic inflammation have demonstrated a similar codependency of platelet and neutrophil recruitment in inflamed postcapillary venules.7,27 For example, Vowinkel et al7 have reported that the adhesion of platelets in colonic venules of mice with colitis is profoundly reduced when the animals are rendered neutropenic with antineutrophil serum. Another manifestation of the neutrophil-platelet interactions that occur during chronic inflammation is the formation of platelet-leukocyte aggregates in circulating blood.28 The results of our study indicate that such aggregates are generated in blood during chronic Ang II exposure and that platelets preferentially form aggregates with neutrophils, which supports the contention that neutrophils are an important target of Ang II action.

Although the ability of Ang II to elicit oxidative stress in endothelial cells is well documented,22,29 less is known about the effects of the peptide in promoting ROS production in leukocytes. Monocytes from hypertensive patients and neutrophils from hypercholesterolemic patients exhibit increased ROS production in response to Ang II stimulation.30 Furthermore, it has been demonstrated that Ang II enhances NADPH oxidase activity in human neutrophils by engaging with AT1Rs and by activating mitogen-activated protein kinase, calcineurin, and the transcription factor nuclear factor-κB.31 These observations are consistent with our finding that neutrophil-associated NADPH oxidase makes a significant contribution to the oxidative stress that is manifested in the walls of postcapillary venules exposed to elevated levels of Ang II.

Perspectives

Ang II is known to mediate the proinflammatory and prothrombogenic phenotype that is assumed by the microvasculature in the presence of risk factors for cardiovascular disease, including HTN and hypercholesterolemia. These responses to Ang II have been linked to the ability of the peptide to induce a pro-oxidative state in endothelial cells via AT1Rs, mediated activation of NADPH oxidase. The findings of the present study suggest that the neutrophils adhering to the vessel wall in response to Ang II also undergo an oxidative burst via AT1Rs. Furthermore, it appears that the neutrophil-derived ROS is of greater quantitative importance than endothelial cell-derived ROS production in eliciting the inflammatory and prothrombogenic phenotype associated with elevated Ang II levels. These findings suggest that neutrophilic NADPH oxidase may be a novel therapeutic target for prevention of the inflammatory and thrombogenic responses that accompany the risk factors for cardiovascular diseases.

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Disclosures

None.
References


Novelty and Significance

What Is New?

- The link among Ang II receptor activation, oxidative stress, and microvascular inflammation appears to involve the activation of NADPH oxidase in circulating blood cells.
- Neutrophils, but not platelets, are largely responsible for the oxidative stress associated with chronically elevated Ang II levels.

What Is Relevant?

- Leukocyte activation is equally as important as endothelial cell activation in mediating the chronic, low-grade inflammation that accompanies Ang II–mediated HTN.

- Neutrophil-associated NADPH oxidase represents a novel therapeutic target for prevention of the inflammatory and thrombogenic responses that accompany HTN and other risk factors for cardiovascular diseases.

Summary

Chronic Ang II infusion induces oxidative stress and promotes the recruitment of adherent leukocytes and platelets in skeletal muscle venules. Neutrophils, but not platelets, appear to contribute to the oxidative stress via a mechanism that involves leukocyte-associated AT1rs and NADPH oxidase. This mechanism is also critical in mediating the blood cell-endothelial cell interactions elicited by Ang II.
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Materials and Methods

Production of bone marrow chimeras: Bone marrow cells were isolated from the femurs and tibias of donor mice. WT→WT chimeras were produced by transplanting marrow from WT C57BL/6 mice expressing CD45.2 into WT congenic recipients that express CD45.1. gp91phox-/→WT, and AT1r-/→WT mice were generated by transplanting marrow from either gp91phox-/ or AT1r-/ mice (both of which express CD45.2) into WT (CD45.1) recipients. WT→gp91phox-/ mice were also generated by transplanting bone marrow from WT mice into gp91phox-/ recipients. Recipient mice were irradiated with two doses of 500-525 Rads, 3 h apart, after which 5 x 10^6 donor bone marrow cells were injected into the femoral vein. The chimeras were kept in autoclaved cages, with 0.2% neomycin in drinking water for two weeks, after which normal drinking water was used. Flow cytometry used to verify chimera reconstitution (usually requiring 6-8 weeks) by staining for CD45.1 (CD45 congenic mice) and CD45.2 (expressed by C57BL/6 mice or knockout mice on C57BL/6 background) expression on circulating leukocytes with a FITC-conjugated anti-CD45.2 antibody and a PE-conjugated anti-CD45.1 antibody (eBioscience). This procedure normally yields greater than 90% penetrance of the transferred marrow at 6 weeks or longer after transplant. These bone marrow transfer protocols allow for the creation of mice wherein the genetic deficiency (gp91phox and AT1r) is confined to the circulating blood cells.

Intravital microscopic methods: Ketamine/xylazine anesthetized mice were placed in a semi-upright position on a Plexiglas microscope stage. Then, 100 x 10^6 CFSE-labeled platelets were infused over 5 min and were allowed to circulate for 5 min before intravital observation. Venules with diameters between 20 – 40 μm and wall shear rates >500 sec^-1 were selected for study. An optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX) was used to measure red blood cell velocity (V_{RBC}) in the microvessels and wall shear rate (WSR) was calculated from the product of mean red blood cell velocity [V_{mean} = V_{RBC}/1.6] and microvascular cross-sectional area, assuming cylindrical geometry. Wall shear rate (WSR) was calculated on the
basis of the Newtonian definition: \( WSR = 8(V_{\text{mean}}/D_v) \). Adherent leukocytes in postcapillary venules were determined, over a 2 min period, by light microscopy, while the adhesion of CFSE-labelled platelets was monitored under fluorescence microscopy for a period of 1 min. The microscopic images were recorded on DVD and blood cell adhesion was analyzed offline. Adhesion values were recorded in 100 µm length vessel segments at 300 µm intervals along the length of the vessel, beginning as near to the source of the venule as possible. The mean value of each variable within a single venule was calculated, and comparisons were made between the experimental groups. Platelets (number per millimeter square) were considered adherent if they arrested for ≥30 s. A leukocyte was considered adherent if it remained stationary for ≥30 s (number per millimeter square). Leukocyte emigration was measured online at the end of each observation period and expressed as the number of interstitial leukocytes per high-powered field of view adjacent to the observed vascular segment (number per field).

**Flow cytometric analysis of leukocyte subpopulations:** Different circulating leukocyte populations were monitored using flow cytometry. Mouse blood was collected by tail-vein bleed and mixed with heparin (20U/mL). The erythrocytes were lysed, and leukocytes were pelleted by centrifugation, resuspended in FACS buffer. Samples were first incubated with FcR block (CD16/CD32), and then stained with a combination of 6 antibodies to identify total leukocytes (CD45.2-FITC), monocytes (CD115-PE), B-lymphocytes (CD45R-PE/Cy5), T-lymphocytes (CD3-PE/Cy7), neutrophils (Gr-1-eFluor660), and macrophage (F4/80-eFluor450) (all from eBioscience). Appropriate isotype controls were used to determine non-specific binding. Gating was performed for live, CD45.2+ events constituting the total leukocyte population.

Four-color flow cytometry utilizing a combination of CD41-APC, CD45.2-FITC, Gr-1-PE, F4/80-eFluor450 and isotype control (eBioscience) were used to detect and quantify aggregates of leukocytes, neutrophils, and monocytes with platelets. Neutrophils and monocytes were distinguished from other cells by their size and complexity. All samples were analyzed on BD LSR II (BD Biosciences). Data analysis was performed using BD FACS Diva software (version 6.0).

**Spinning disk confocal microscopy:** An Olympus BX51WI upright microscope (Olympus, Center Valley, PA) with a 40XW (LUMPLFLN) objective and equipped with a 3i LaserStack laser launch (3i; Denver, CO), Yokogawa CSU-X1-A1N-E spinning disk confocal unit (Yokogawa Electric Corporation, Tokyo, Japan) and electron multiplier CCD camera (C9100-13; Hamamatsu, Bridgewater, NJ) was used for these experiments. Anti-mouse CD45.2 FITC or AF568 conjugated (4 µg/mouse; eBiosciences; San Diego, CA), anti-mouse CD115 (10 µg/mouse; Biolegend; San Diego, CA) conjugated with Alex Fluor 568 protein labeling kit (Molecular Probe; Eugene, OR) and anti-mouse Ly-6G (Gr-1) eFluor 660 or AF488 conjugated (2 µg/mouse; eBiosciences), anti-mouse CD3 e660 (2 µg/mouse; eBiosciences) and anti-mouse B220 e660 (2 µg/mouse; eBiosciences) were injected intravenously to image total leukocytes, monocytes and neutrophils, T-lymphocytes and B-lymphocytes, respectively. 488-, 561 and 640-nm laser excitation were used in rapid sequence and visualized with the appropriate filters (Semrock, Rochester, NY). Typical exposure times for all excitation wavelengths were 200 ms. Slidebook software (3i Denver, CO) was used to drive the confocal system and capture images for offline analysis. Leukocytes were considered adherent if they remained stationary for 30 seconds or longer.
Results

Figure S1. Fluorescence microscopic images of DHR oxidation within and surrounding cremaster muscle venules in the following experimental groups: WT controls (panel A), WT-AngII (panel B), angiotensin II-type 1 receptor deficient (AT1r−/−)-AngII (panel C), WT-neutropenic-AngII (panel D), WT-thrombocytopenic-AngII (panel E) and gp91phox deficient mice (gp91phox−/−)-AngII (panel F) mice.
Figure S2. Panel A: Spinning-disk confocal-microscopic imaging of different leukocyte populations recruited into cremaster postcapillary venules of AngII treated mice. In-vivo Ab labeling was used to identify adherent CD45+ cells (green) A1, CD115+ monocytes (blue) A2, Gr-1+ neutrophils (red) A3 and CD45, CD115, Gr-1 positive cells (multi-channel overlay) A4. Panel B: Number of cells in each leukocyte subset recruited into cremaster muscle postcapillary venules of AngII treated mice (per mm²). CD45 represents total leukocyte population, CD115 shows monocytes, Gr-1 represents neutrophils, CD3 represents T-lymphocytes and B220 corresponds to B-lymphocytes. *, indicates p<0.05 relative to CD45+ cells.
Figure S3. Formation of platelet aggregates with neutrophils (PNA), monocytes (PMA) and lymphocytes (PLyA) in WT and AngII treated mice, as detected by flow-cytometry. CD41 MFI indicates the relative amount of platelets attached per leukocyte. *, indicates p<0.05 relative to WT control.