Leukocyte–Endothelial Cell Adhesion in Spontaneously Hypertensive and Normotensive Rats

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Although the etiology of hypertension-related organ damage remains poorly understood, it has recently been proposed that activated and adherent leukocytes may contribute to the pathogenesis of progressive organ injury in hypertension. The objective of this study was to determine whether the adherence and emigration of leukocytes in microvessels differ between spontaneously hypertensive and normotensive rats. Leukocyte adherence, rolling, and emigration as well as vessel diameter and erythrocyte velocity were monitored in mesenteric venules of age-matched normotensive and hypertensive rats. Measurements were obtained under baseline conditions and during superfusion of the mesentery with either platelet activating factor, leukotriene B4, or Nω-nitro-L-arginine-methyl ester, an inhibitor of nitric oxide synthesis. Tissue-associated myeloperoxidase activity, an index of the total tissue granulocyte population, was measured in various tissues of normotensive and hypertensive rats. Systemic arterial pressure and the circulating polymorphonuclear leukocyte count were elevated in hypertensive relative to normotensive rats. The number of adherent and emigrated leukocytes under baseline conditions did not differ between normotensive and hypertensive rats. Although the nitric oxide synthase inhibitor caused a similar rise in leukocyte adherence and emigration in both rat strains, the adhesive interactions elicited by either platelet activating factor or leukotriene B4 were significantly blunted in hypertensive relative to normotensive rats. Flow cytometric analysis of whole-blood samples revealed a lower surface expression of CD11b/CD18 on leukocytes from hypertensive rats under stimulated conditions. Myeloperoxidase activity in mesentery and small and large intestine was low, whereas lung, spleen, and stomach values were high in hypertensive compared with normotensive rats. These results indicate that the altered leukocyte distribution in spontaneously hypertensive rats may result from a reduced capacity for leukocytes to adhere and emigrate in response to endogenous inflammatory stimuli such as leukotriene B4 and platelet activating factor.

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KEY WORDS • platelet activating factor • leukotrienes B • nitric oxide • leukocytes • endothelium

The role of circulating leukocytes in acute and chronic inflammatory disorders is well recognized. Recently, these cells have received much attention as possible mediators of the endothelial cell injury and microvascular dysfunction associated with a number of cardiovascular diseases, including ischemic disorders1-3 and atherogenesis.4 Leukocytes have also been implicated in the pathophysiology of hypertension. Schmid-Schönbein and coworkers5 have proposed that hypertension-induced organ damage in the spontaneously hypertensive rat (SHR) might be related to the elevated blood leukocyte count and the greater proportion of spontaneously activated granulocytes circulating in these rats compared with normotensive Wistar-Kyoto (WKY) rats. Based on these observations, the authors proposed that the large population of activated leukocytes may be accompanied by increased leukocyte accumulation in tissues as a consequence of either capillary plugging or adherence in postcapillary venules. The latter possibility seems tenable inasmuch as granulocyte activation is normally associated with an increased surface expression of adhesion glycoproteins on leukocytes.6 Activated neutrophils could also promote leukocyte adherence via superoxide-mediated inactivation of nitric oxide, which appears to be an endogenous endothelial cell-derived inhibitor of leukocyte adhesion in postcapillary venules.7 Studies of endothelium-dependant relaxation of aortic strips suggest that there is a reduction in the basal production of nitric oxide by endothelial cells in blood vessels from hypertensive rats.8,9 Thus, decreased production of nitric oxide might contribute to the elevated blood pressure as well as an increased leukocyte–endothelial cell adhesion in SHRs. Although leukocyte accumulation in tissues is an integral component of the hypothesis implicating activated leukocytes in the pathogenesis of hypertension, it remains uncertain whether leukocyte–endothelial cell adhesion is altered in SHR relative to WKY rats. Thus, the major objective of this study was to determine if...
basal and stimulated leukocyte adherence, emigration, or both differ between the microvasculature of SHR and WKY rats. Additional objectives were to determine whether 1) the surface expression of CD11b/CD18 on unstimulated and stimulated neutrophils differs between SHR and WKY rats, 2) the magnitude of leukocyte–endothelial cell adhesion induced by inhibition of nitric oxide synthesis differs between SHR and WKY rats, and 3) the tissue distribution of granulocytes (as assessed by tissue myeloperoxidase activity) is altered in SHR relative to WKY rats.

**Methods**

**Animal Preparation**

The surgical procedures on animals used in these experiments were in accordance with institutional guidelines. Thirty male WKY rats and 23 SHR (Harlan Sprague Dawley, Frederick, Md.) weighing 160–240 g (6–9 weeks of age) and fasted 12–18 hours before the experiments were anesthetized with 120 mg/kg Inactin [Na-5-ethyl-l(l'-methyl-propyl)2-thio-barbiturate, Byk Gulden, Konstanz, FRG]. A tracheotomy was performed, and the left carotid artery was cannulated for continuous measurement and recording of systemic blood pressure and heart rate (Statham P23A transducer, Oxnard, Calif.; Grass recorder, Grass Instruments, Quincy, Mass.). Samples of 0.3 mL blood were obtained after the preparations were stabilized for total leukocyte and neutrophil counts on a hemocytometer using an acetic acid–crystal violet stain.

**Intravital Microscopy**

Animals were placed in a supine position on an adjustable Plexiglas microscope stage, and an exteriorized segment of midjejunum was draped over an optically clear viewing pedestal, allowing transillumination of a 2-cm² section, as described previously. The exposed bowel wall was draped with saline-soaked gauze, and the mesentery was covered with plastic wrap. Thirty male WKY rats and 23 SHR (Harlan Sprague Dawley, Frederick, Md.) weighing 160–240 g (6–9 weeks of age) and fasted 12–18 hours before the experiments were anesthetized with 120 mg/kg Inactin [Na-5-ethyl-l(l'-methyl-propyl)2-thio-barbiturate, Byk Gulden, Konstanz, FRG]. A tracheotomy was performed, and the left carotid artery was cannulated for continuous measurement and recording of systemic blood pressure and heart rate (Statham P23A transducer, Oxnard, Calif.; Grass recorder, Grass Instruments, Quincy, Mass.). Samples of 0.3 mL blood were obtained after the preparations were stabilized for total leukocyte and neutrophil counts on a hemocytometer using an acetic acid–crystal violet stain.

The images were recorded with a videocassette recorder (Hitachi WK-C150) mounted on the microscope projected the image onto a color monitor (Sony PVM-2030, Japan). The images were recorded with a videocassette recorder (Panasonic NV8950, Japan) for playback analysis. Venuolar diameter (Dv) and arteriolar diameter (Da) were measured either on- or off-line with a video image-shearing monitor (IPM, Inc., La Mesa, Calif.). Blood cell centerline velocity was measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, Tex.). Mean red blood cell velocity (Vmean) was calculated assuming Vmean = centerline velocity/1.6. Venular shear rate (γ) was calculated based on the Newtonian definition:

\[ \gamma = 8(V_{\text{mean}}/D_{v}) \]

The number of adherent and emigrated leukocytes was determined during playback of videotaped images. A leukocyte was defined as adherent to venular endothelium if it remained stationary for at least 30 seconds. Leukocyte adherence was expressed as the number per 100-μm length of venule. The number of emigrated leukocytes was also determined off-line during playback of videotaped images. Any interstitial leukocytes present in the mesentery at the onset of the experiment were subtracted from the total number of leukocytes that accumulated during the course of the experiment. Rolling leukocytes were defined as white blood cells moving at a slower velocity than erythrocytes in the same vessel. The leukocyte rolling velocity (Vro) was determined from the time required for a leukocyte to move along a 100-μm length of the microvessel. A mean of 10 estimates of transit time was used to calculate Vro.

**Experimental Protocols**

When arterial pressure and red blood cell velocity were stable during superfusion with BBS, images of the mesenteric preparation were recorded for 10 minutes. Then the superfusion buffer was changed to either 100 mmol/L platelet activating factor (PAF), 20 mmol/L leukotriene B₄ (LTB₄), or 100 μmol/L N⁵-nitro-L-arginine-methyl ester (L-NAME), each dissolved in BBS. The mesentry was superfused with the PAF- or LTB₄-containing test solution for 40 minutes, whereas L-NAME was superfused for 70 minutes, with video recordings in all instances taken during the final 10 minutes of superfusion. This general protocol was applied to both SHR and WKY rats.

In some experiments, tissue samples of stomach, duodenum, jejunum, ileum, colon, mesentery, lung, heart, liver, spleen, and kidney were rapidly excised, rinsed with ice-cold saline, blotted dry, and frozen at −70°C until thawing for measurement of myeloperoxidase activity, an index of the number of tissue granulocytes. Thawed tissue was homogenized (10% wt/vol) in K-, buffer (20 mmol/L, pH 7.4) and centrifuged (8,000 rpm, 4°C, 20 minutes). The pellet was rehomogenized in an equivalent volume of 50 mmol/L KCl, buffer containing 0.5% hexadecyltrimethylammonium bromide, sonicated, and centrifuged. The supernatant was used for determination of tissue myeloperoxidase activity. Myeloperoxidase activity was assessed by measuring the H₂O₂-dependent oxidation of 3,3',5,5'-tetramethylbenzidine, resulting in a blue chromogen detectable spectrophotometrically at 655 nm. One unit of enzyme activity is defined as the amount of myeloperoxidase present that causes a change in absorbance of 1.0/min at 37°C. In rats, one unit of myeloperoxidase activity is equivalent to 10⁶ neutrophils.

**Flow Cytometry Studies**

Flow cytometry was used to quantitate basal and stimulated surface expression of CD11b/CD18 on polymorphonuclear leukocytes from SHR and WKY rats. The analysis was performed with whole-blood samples to minimize artifactual upregulation of CD11b/CD18 associated with ex vivo manipulation. A 90-μL aliquot of heparinized whole blood was incubated either with...
zymosan-activated plasma (10 μL) or with 10 μL phosphate-buffered saline (PBS) for 15 minutes at 37°C. After incubation, 10 μL (10 μg/mL) of a solution containing a monoclonal antibody directed against rat CD11b (mAb C117, provided by Dr. Robert Todd) was added to samples and incubated at room temperature for 15 minutes. The samples were then washed twice with PBS and centrifuged at 1,000 rpm for 3 minutes. The red cells were then lysed by addition of Immuno-lyse reagent (Coulter Immunology, Hialeah, Fla.). After vigorous vortexing, the samples were washed twice with PBS, centrifuged, and resuspended in 1% paraformaldehyde. Analysis was performed on an EPICS 753 flow cytometer/sorter (Coulter Electronics, Hialeah, Fla.) for the simultaneous accumulation of immunofluorescence and forward-angle and 90° light scatter signals. Dead cells and debris were excluded by forward-angle and 90° light scatter gating or, in some experiments, by the exclusion of dead cells that incorporate propidium iodide. Controls included cells stained with the secondary antibody alone, and this information was used to determine the percent of cells reacting with the primary antibody. Five thousand cells were analyzed in each experiment, and each experiment was performed on blood from four SHR and WKY rats.

Statistical Analyses
All data were analyzed with standard statistical analysis, i.e., analysis of variance with Scheffé's (post hoc) test. All values are expressed as mean±SEM, and statistical significance was set at a value of $p<0.05$.

Results

Figure 1 summarizes the leukocyte adherence responses in SHR and WKY mesenteric venules that were exposed to either 100 nmol/L PAF, 20 nmol/L LTB₄, or 100 μmol/L L-NAME. Under baseline conditions, the number of adherent leukocytes in SHR (2.30±0.48 per 100 μm length) tended to be lower than that observed in WKY rats (3.23±0.47); however, this difference did not reach statistical significance ($p=0.09$). In WKY rats, PAF, LTB₄, and L-NAME were all very effective in promoting leukocyte adherence in mesenteric venules, as previously shown in Sprague-Dawley rats and cats. Although PAF and LTB₄ also elicited leukocyte adherence in SHR, the magnitude of the adherence responses was significantly blunted relative to those observed in WKY rats. In contrast, inhibition of nitric oxide synthesis with L-NAME produced a similar adherence response in SHR and WKY rats. The pattern of leukocyte emigration responses elicited by the three agents was similar to that observed with leukocyte adherence (Figure 2). The resting number of emigrated leukocytes in SHR was nearly significantly different ($p=0.052$) from that of WKY rats. Although the leukocyte emigration response to PAF in SHR was significantly lower than in WKY rats, no statistical differences between normotensive and hypertensive animals were noted for either LTB₄ or L-NAME.

Leukocyte rolling velocity ($V_{\text{sc}}$) (Figure 3) was significantly higher in mesenteric venules of SHR than of WKY rats under baseline conditions. Neither PAF nor LTB₄ altered $V_{\text{sc}}$ in both SHR and WKY rats. Superoxide with L-NAME reduced $V_{\text{sc}}$ in mesenteric venules of hypertensive rats by approximately 50%, yet the nitric oxide synthesis inhibitor did not affect $V_{\text{sc}}$ in the normotensive WKY rats.

Venular wall shear rate in SHR mesentery was significantly higher than that observed in WKY mesentery.
under baseline conditions (Figure 4). This relation remained largely unchanged during superfusion with PAF and LTB₄. L-NAME, however, reduced the wall shear rate in SHR by approximately 50%, but it did not affect shear rate in normotensive rats. Neither PAF, LTB₄, nor L-NAME altered venular diameter; however, L-NAME caused a significant reduction (17.2%) in the diameter of mesenteric arterioles from WKY rats (from 19.0±3.5 to 15.7±2.5 μm) but not from SHR (from 18.5±2.6 to 16.8±4.1 μm). There was no significant difference between resting venular diameters of WKY (30.3±1.3 μm) and SHR (35.0±1.8 μm) arterioles. Systemic arterial blood pressure in SHR (131±4.4 mm Hg) was significantly greater than values obtained in WKY rats (94±2 mm Hg). None of the test substances altered arterial pressure.

The total leukocyte count did not differ between WKY rats (5.1±0.3 per 10⁹/L) and SHR (5.18±0.34 per 10⁹/L); however, there was a significantly higher number of circulating neutrophils in SHR (1.17±0.12 per 10⁹/L) compared with WKY rats (0.83±0.07 per 10⁹/L). Figure 5 compares the tissue-associated myeloperoxidase activity in 11 different organs of WKY rats and SHR. In lung, spleen, and stomach, tissue myeloperoxidase activity was higher in SHR than WKY rats, whereas myeloperoxidase activity was lower in the duodenum, ileum, and colon of SHR.

Flow cytometric analysis of CD11b/CD18 (Mac-1) expression on isolated rat neutrophils revealed that basal expression of this adhesion glycoprotein was higher (p=0.06) in WKY rats (22.3±3.0%) than in

FIGURE 2. Bar graph shows effects of platelet activating factor (PAF), leukotriene B₄ (LTB₄), and N⁰-nitro-L-arginine-methyl ester (L-NAME) on leukocyte emigration in mesenteric venules of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). *p<0.05 relative to control value; +p<0.05 relative to corresponding normotensive group.

FIGURE 3. Bar graph shows effects of platelet activating factor (PAF), leukotriene B₄ (LTB₄), and N⁰-nitro-L-arginine-methyl ester (L-NAME) on leukocyte rolling velocity in mesenteric venules of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). *p<0.05 relative to control value; +p<0.05 relative to corresponding normotensive group.
SHR (15.3±1.7%). This difference was more pronounced when the neutrophils were exposed to zymosan-activated serum; i.e., fluorescence intensity of CD11b binding in neutrophils from SHR (28.1±3.2%) was significantly lower than that elicited in neutrophils from WKY rats (41.3±1.2%).

Discussion

A growing body of evidence indicates that polymorphonuclear leukocytes contribute to the pathobiology of a variety of cardiovascular diseases, including myocardial infarction, stroke, intestinal ischemia, and circulatory shock. Although most of the data supporting a role for leukocytes in cardiovascular disease has been derived from animal experimentation, epidemiological studies on patients also indicate that the circulating leukocyte count can be used as a predictive indicator of the risk of myocardial infarction and stroke. An elevated blood leukocyte count in SHR relative to normotensive controls has recently provided the basis for implicating leukocytes in the pathophysiology of arterial hypertension. Schmid-Schönbein and associates have proposed that hypertension-induced organ damage in SHR might be related to both an elevated blood leukocyte count and a greater proportion of spontaneously activated granulocytes circulating in these rats compared with normotensive WKY rats. These authors also proposed that the activated circulating leukocytes may lead to an altered leukocyte distribution in tissues as a consequence of either capillary plugging or adherence in postcapillary venules.

The results of the present study support the contention that SHR exhibit an altered distribution of granulocytes both within the vasculature and between tissues relative to WKY rats. The 6–9-week-old SHR used in our studies were characterized (relative to WKY rats) by 1) an elevated circulating blood granulocyte count, 2) a lower level of surface expression of CD11b/CD18 on leukocytes under stimulated conditions, 3) reduced leukocyte adherence and emigration in response to inflam-
matory stimuli (PAF and LTB₄), and 4) an increased tissue granulocyte level in lung, spleen, and stomach, with a reduced tissue level in the small and large bowels. Although inhibition of nitric oxide synthesis resulted in a greater reduction in mesenteric arteriolar diameter in WKY rats than in SHR, no differences in the magnitude of L-NAME-induced leukocyte adherence and emigration were noted between the two strains. Our observation that postcapillary venules in SHR exhibit an attenuated leukocyte adherence and emigration response to inflammatory mediators relative to WKY may explain the greater number of circulating granulocytes in SHR.

The greater tendency for leukocytes to “demarginate” in SHR may be explained by a variety of factors. One explanation, which is supported by our results, is that leukocytes in SHR have a reduced capacity to mobilize CD11b/CD18 to the cell surface in response to an inflammatory stimulus. It is conceivable that SHR also exhibit a lower level of expression of endothelial cell adhesion glycoproteins such as ICAM-1, E-selectin, and/or P-selectin in postcapillary venules; however, this possibility has not been directly addressed. The blunted leukocyte–endothelial cell adhesive interactions elicited by PAF and LTB₄ in SHR may also result from an altered affinity or number of receptors or both on granulocytes for the inflammatory mediators. Such an explanation is consistent with reports of a reduced number of glucocorticoid receptors on mononuclear cells from SHR. A final consideration in accounting for the reduced leukocyte adherence in mesenteric venules of SHR is venular shear rate. Previous reports indicate that graded reductions in venular shear rate can result in progressive increases in the number of adherent leukocytes. Although venular shear rate was generally lower in WKY rats than SHR, these reduced shear rates were not sufficiently low (increased leukocyte adherence is observed at shear rates <250 sec⁻¹) to promote adherence in mesenteric venules.

Although the reduced capacity of neutrophils in SHR to mobilize CD11b/CD18 to the cell surface in response to an inflammatory stimulus may explain the neutrophilia repeatedly observed in these animals, it appears inconsistent with previous reports of an increased level of activated neutrophils in the blood of SHR. The production of reactive oxygen metabolites by activated neutrophils is often associated with an upregulation or production of reactive oxygen metabolites by activated neutrophils. Although venular shear rate was generally lower in WKY rats than SHR, these reduced shear rates were not sufficiently low (increased leukocyte adherence is observed at shear rates <250 sec⁻¹) to promote adherence in mesenteric venules.

In the present study, we reasoned that if nitric oxide production is lower in SHR than in WKY rats, then the leukocyte adherence responses to inhibition of nitric oxide synthesis should be greater in WKY rats. Although L-NAME elicited a dramatic increase in both leukocyte adherence and emigration in mesenteric venules of WKY rats, the responses in venules of SHR were nearly identical. These observations suggest either that the ability of vascular endothelium to produce nitric oxide does not differ between SHR and WKY rats or that such differences are exhibited in the arterial but not the venous segment of the microvasculature. Our finding that L-NAME induced a greater reduction in arteriolar diameter in WKY rats than SHR supports the latter possibility.

An interesting observation in the present study was the differences in tissue distribution of granulocytes between SHR and WKY rats. We noted a greater accumulation of peroxidase-positive cells in lung, spleen, and stomach of SHR. Although inhibition of nitric oxide synthesis resulted in a greater reduction in mesenteric arteriolar diameter in SHR than WKY rats, no differences in nitric oxide production were noted between the two strains. Our observation that postcapillary venules in SHR exhibit an attenuated leukocyte adherence and emigration response to inflammatory mediators relative to WKY may explain the greater number of circulating granulocytes in SHR.

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