Contribution of Fluid Shear Response in Leukocytes to Hemodynamic Resistance in the Spontaneously Hypertensive Rat

Shunichi Fukuda, Takanori Yasu, Nobuhiko Kobayashi, Nahoko Ikeda, Geert W. Schmid-Schönbein

Abstract—The mechanisms for elevation of peripheral vascular resistance in spontaneously hypertensive rats (SHR), a glucocorticoid-dependent form of hypertension, are unresolved. An increase in hemodynamic resistance caused by circulating blood may be a factor. Physiological fluid shear stress induces a variety of responses in circulating leukocytes, including pseudopod retraction. Due to high rigidity, leukocytes with pseudopods have greater difficulty to pass through capillaries. Because SHR have more circulating leukocytes with pseudopods, we hypothesize that inhibition of the leukocyte shear response by glucocorticoids in SHR impairs normal leukocyte passage through capillaries and causes enhanced resistance in capillary channels. Fluid shear leads to retraction of pseudopods in normal leukocytes, whereas shear induces pseudopod projection in SHR and dexamethasone-treated Wistar rats. The high incidence of circulating leukocytes with pseudopods results in slower cell passage through capillaries under normal blood flow and during reduced flow enhanced capillary plugging both in vivo and in vitro. SHR blood requires higher pressure (90.0±8.2 mm Hg) than Wistar Kyoto rat (WKY, 69.6±6.5 mm Hg; P<0.0001) or adrenalectomized SHR (73.5±2.1 mm Hg; P=0.0009) at the same flow rate in the resting hemodynamically isolated skeletal muscle microcirculation. Intravenous injection of blood from SHR, but not WKY, causes blood pressure increase in normal rats, which depends on pseudopod formation. We conclude that in addition to enhanced vascular tone, pseudopod formation with lack of normal fluid shear response may serve as mechanisms for an elevated hemodynamic resistance in SHR. 

Key Words: capillaries ■ glucocorticoids ■ pseudopod formation ■ mechotransduction

There is a widely held opinion that elevation of blood pressure and peripheral resistance in arterial hypertension is due to an increase in vasoconstriction and restructuring of arteries and arterioles. But no conclusive evidence exists that demonstrates that the shift in resistance of arteries/arterioles is actually the sole cause for the blood pressure elevation. In addition to vascular factors, fluid stresses in circulating blood may contribute to vascular resistance elevation. Perhaps most surprisingly, the relatively small number of leukocytes in the circulation has a powerful effect on the hemodynamic resistance in capillaries with single file cells. Because leukocytes are larger and less deformable than erythrocytes, leukocytes move slower through capillaries. Erythrocytes are forced to slow down, disturbing their position in the capillary lumen and sharply increasing their apparent viscosity. The reduced leukocyte velocity causes an increase in microvascular resistance produced by erythrocytes. The effect depends on leukocytes biomechanical properties, it requires no adhesion to the endothelium and can also be simulated by microspheres with similar dimensions as leukocytes and with similar low numbers.

An important aspect of this mechanism relates to the ability of leukocytes to form pseudopods. Because the resting diameter of leukocytes is larger than most capillary diameters, leukocytes are required to deform in most capillary networks. The deformation depends on cell mechanical properties and as rigid structures in the cell cytoplasm is strongly influenced by pseudopods.

Furthermore, we have recently demonstrated that physiological levels of fluid shear promote pseudopod retraction and keep circulating leukocytes in a spherical shape. Continued shear exposure contributes to formation of passive spherical cell shapes without pseudopods. Therefore, impairment of the shear response may cause an enhanced number of leukocytes with pseudopods, slowing of leukocyte passage through capillaries, and elevated microvascular resistance.

SHR have an abnormal response to glucocorticoids. SHR have an elevated leukocyte count, reduced number of leukocyte rolling on and adhering to venules due to P-selectin...
suppression, an impaired dilation of vascular smooth muscle, enhanced oxidative stress, and apoptosis, all of which are mediated by glucocorticoids. By chance, we recently observed that glucocorticoids have a profound effect on shear response of circulating leukocytes.19

Thus, we hypothesize that glucocorticoid reverses the leukocyte shear response in SHR, which causes an increase in the counts of leukocytes with pseudopods. The effect leads to impaired passage through single file capillaries and elevated hemodynamic resistance.

We present a sequence of in vivo and in vitro studies designed to explore key elements of the circulatory consequences of reversed shear response in leukocytes of SHR and dexamethasone-treated rats. The results suggest that, in addition to enhanced vascular tone, a shear-mediated hemorheological mechanism in capillaries may contribute to the elevated peripheral resistance in SHR.

Materials and Methods

Leukocyte separation by centrifugation was avoided because it impairs the shear response.21 For an expanded Materials and Methods section, see the online data supplement available at http://circres.ahajournals.org.

Animals

After general anesthesia (somotropin, 50 mg/kg), the femoral veins and arteries of mature male Wistar rats (n=100), SHR (n=50), Wistar Kyoto rats (WKY, n=29), adrenalectomized Wistar rats (n=4), adrenalectomized SHR (n=7), and adrenalectomized WKY (n=4) (290 to 390 g; Charles River Laboratories, Wilmington, Mass) were cannulated. Mean blood pressure was measured in the femoral artery (MABP). Adrenalectomized animals were purchased from the breeder and received 0.9% saline in the intravenous pump (Harvard Apparatus) and the draining vein held at 0 mm Hg. Plasma-Lyte (Baxter Health Care) with 5% bovine serum albumin (Sigma), 10 U/mL ammonium heparin, and 0.015% papaverine hydrochloride was perfused to fully dilate the microvessels.19

Blood (30 U/mL ammonium heparin) was collected from either Wistar, DX-Wistar, SHR, WKY, or adrenalectomized SHR. The blood (0.1 mL, CFSE; Molecular Probes) served as label for circulating leukocytes.

Leukocyte Kinetics in the Rat Microcirculation

The mesenteric microcirculation was visualized through an intravital fluorescence microscope.9–11 Carboxyfluorescein succinimidyl ester (1.0 mg/kg, CFSE; Molecular Probes) served as label for circulating leukocytes.

Relative Transit Time in Mesenteric Microcirculation

Relative transit time (leukocytes/erythrocytes transit time) of CFSE-labeled leukocytes from WKY, SHR, Wistar rats, and Wistar rats treated with 0.5 mg/kg dexamethasone for 7 days (DX-Wistar) was determined in selected true capillaries at constant MABP. CFSE-labeled leukocytes in whole blood from either WKY or SHR (or normotensive Wistar or DX-Wistar) were observed for 10 minutes after injection. When no more labeled cells were encountered, a second aliquot of blood was administered. In the same capillary network, the transit time of 50 PKH-26-labeled (Sigma) erythrocytes of the recipient rat and 30 leukocytes of each donor rat was measured.20

In Vivo Leukocyte Kinetics in Capillaries During Reduced Flow

The velocity in mesenteric capillaries in Wistar or DX-Wistar was reduced to 0.2 to 1.5 mm/sec by partial occlusion of the celiac artery using an extravascular balloon (Medtronic PS Medical). PKH-26-labeled erythrocytes were used to measure velocity. The number of plugging leukocytes per capillary in each observation field was determined. “Plugging leukocytes” were defined as cells that obstruct a capillary for at least 30 seconds.

Measurement of Pressure-Flow Relation in Rat Gracilis Muscle

The gracilus muscle of normotensive Wistar was hemodynamically isolated from the central circulation using microsurgery.14–20 The feeder was connected to a precise syringe pump (Harvard Apparatus) and the draining vein held at 0 mm Hg. Plasma-Lyte (Baxter Health Care) with 5% bovine serum albumin (Sigma), 10 U/mL ammonium heparin, and 0.015% papaverine hydrochloride was perfused to fully dilate the microvessels.21

Blood (30 U/mL ammonium heparin) was collected from either Wistar, DX-Wistar, SHR, WKY, or adrenalectomized SHR. The blood (0.1 mL, CFSE; Molecular Probes) served as label for circulating leukocytes.

Shear Response of Suspended Rat Leukocytes in a Cone-and-Plate Device

Arterial blood (30 U/mL ammonium heparin) was collected with or without dexamethasone (Sigma Chemical Corp). Fifty minutes after collection, whole blood was sheared in a cone-and-plate device at a physiological range of 5.0 dyn/cm2 for 10 minutes,10 and immediately fixed with 2% glutaraldehyde. Unsheared samples were fixed simultaneously. The fraction of leukocytes with pseudopods was counted after staining with 0.02% crystal violet.

Shear Response of SHR Leukocytes After Treatment With a β-Blocker

MABP and fraction of leukocytes with pseudopods in arterial blood were examined in SHR 1 hour after treatment with 1 mg propranolol hydrochloride (Inderal) and treatment for 7 days (1 mg/12 hours) and in control SHR (n=4 rats/group).

Shear Response of Leukocytes In Vivo After DX-Treatment

The fraction of leukocytes with pseudopods was examined in Wistar treated with dexamethasone (0.5 mg/kg, 1V) at time 0, Wistar treated with dexamethasone for 7 days (0.5 mg/kg per day, 1M), and in control (n=4/group). In all 3 groups, FMLP (10−9 mol/L) was intravenously injected at time 0. Femoral arterial blood (0.1 mL) was collected every 15 minutes up to 60 minutes and immediately fixed with 2% glutaraldehyde.

Injection of SHR Blood Into Normal Rats

Whole blood with or without 0.1 μmol/L cytochalasin-D from SHR or WKY was injected into normal Wistar (4.5 mL/kg), while MABP was measured. In selected samples, erythrocytes were infused after removal of leukocytes.

MABP After Blood Exchange Between WKY and SHR

Between WKY and SHR (n=5/group), or WKY and WKY (n=6), 10 mL of arterial blood was exchanged every 5 minutes for 5 times, and MABP was measured 5 minutes after each blood exchange.

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Leukocyte Passage Through an In Vitro Microchannel Array

Due to the enhanced propensity for rat blood to coagulate, human blood was used in this study. Human blood was diluted in physiological saline with 1 mmol/L MgSO4 (1:1), and divided into 5 groups: unsheared and sheared controls, 1 μmol/L dexamethasone-treated blood without and with shear, and dexamethasone-treated blood with 5 mmol/L EDTA (Sigma) with shear exposure.

One-minute after blood collection, the sheared groups were exposed to shear stress (5.0 dyn/cm² for 3 or 13 minutes) in a cone-and-plate device. Four, 14, and 19 minutes after blood collection, the passage time of 0.1 mL blood through the Microchannel Flow Analyzer (Kowa Co) was determined. The parallel array of microchannels (3100 channels, equivalent diameter 5 μm, equivalent length 20 μm) was examined under constant suction (15, 20, and 25 cmH₂O). Light microscopic images of the blood passage through the microchannels were recorded on videotape for off-line analysis. Fractions of occluded microchannels due to leukocyte plugging and leukocytes with pseudopods were evaluated 14 minutes after blood collection.

Statistics
Values are shown as mean±SD. Differences were analyzed by ANOVA and Fischer’s PLSD. A value of P<0.05 was considered significant.

Results
In Vitro Shear Response of SHR Leukocytes
The fraction of leukocytes with pseudopods in Wistar was significantly lower with shear than without shear (Figure 1A). In contrast, the number of leukocytes with pseudopods in SHR was significantly increased after shear. Adrenalectomy inhibited the increase in the number of SHR leukocytes with pseudopods after fluid shear. The shear response in WKY was still noticeable, but slightly suppressed compared with that in Wistar (Figure 1A).

With dexamethasone, the shear response in all animal groups was reversed (Figure 1B).

Blood Pressure
MABP in SHR was significantly higher than in Wistar or WKY. Treatment of Wistar with dexamethasone caused a significant rise in MABP (Figure 2A). Adrenalectomy reduced MABP in SHR to normal levels, whereas MABP changed little in Wistar.

In Vivo Pseudopod Formation of Circulating Leukocytes in SHR and DX-Wistar
The average fraction of leukocytes with pseudopods in arterial blood of DX-Wistar was higher than that of control Wistar (Figure 2B). The fraction of cells with pseudopods in SHR (30.4±9.2%) was higher than in WKY (13.8±5.5%) or Wistar. The fraction in WKY was also higher than that in Wistar. Adrenalectomy caused a significant decrease in the fraction of cells with pseudopods in SHR, but not in Wistar (Figure 2B).

Effect of β-Blocker on MABP and Pseudopods in Arterial Blood
Both acute (1-hour) and chronic (7-day) treatment of SHR with a β-blocker, propranolol hydrochloride, significantly reduced MABP; however, there was no change in fraction of circulating leukocytes with pseudopods (Figure 2C).
In all groups, we observed a parallel response between the fraction of leukocytes with pseudopods, fraction of occluded microchannels, and passage time (Figure 3C).

**In Vivo Transit Time of Leukocytes**

The relative transit time in DX-Wistar was significantly longer than that in Wistar (Figure 4A). The average leukocyte transit time in WKY was 1.21±0.06, similar to previous measurements.24 The relative transit time in SHR (1.55±0.18) was significantly longer (Figure 4A).

**Leukocyte Behavior in Capillaries During Reduced Blood Flow**

In Wistar, the fraction of leukocytes plugging capillaries was less than 1% even under low flow velocity, whereas capillary plugging in DX-Wistar was significantly more frequent at reduced flow rates (Figure 4B). Some capillaries were obstructed by leukocytes from DX-Wistar (Figure 4C).

In SHR, most capillaries were easily occluded during reduced flow rates so that the fraction of leukocytes plugging capillaries could not be counted (results not shown).

**Pressure-Flow Relation in Resting Hemodynamically Isolated Gracilis Muscle**

The zero-flow pressure was subtracted from the arterial pressures at each flow21 (Figure 5A). There was no significant difference in zero-flow pressure between groups of blood samples in vasodilated and hemodynamically isolated Wistar skeletal muscle. In each group, the data revealed the same trend (Figure 5B and 5C).

Both in WKY and SHR blood, the pressure-flow curve was shifted equally to the right after addition of erythrocytes to plasma at a hematocrit adjusted to the same value as in whole blood. However, SHR whole blood caused a greater right shift than WKY whole blood. Treatment with cytochalasin-D, to eliminate pseudopods,6 reduced the degree of right shift for SHR whole blood, but not WKY blood (Figure 5B and 5C).

At equal flow (150 µL/min per g), there was no significant difference between WKY and SHR in the perfusion pressure for plasma or plasma with erythrocytes. However, at the same flow the pressure for SHR whole blood was significantly higher than that of WKY whole blood, an effect that was inhibited by cytochalasin-D (Figure 6A). Whole blood from adrenalectomized SHR caused a decrease in pressure compared with that from SHR. Dexamethasone-treatment also induced a rise in pressure of whole blood, but not plasma or plasma with erythrocytes (Figure 6B). The increased pressure due to dexamethasone-treatment was reduced by cytochalasin-D. The pressure with plasma (PPL) or the additional pressure drops due to erythrocytes (PRBC–PPL) were not significantly different between WKY and SHR blood (Figure 6C). In contrast, the additional pressure drop induced by leukocytes (PWB–PRBC) were significantly different in WKY and SHR.

When SHR blood samples were perfused into resting, but not vasodilated, skeletal muscle of SHR, perfusion pressure of whole blood in SHR was significantly higher than that of WKY whole blood, an effect that was inhibited by cytochalasin-D (Figure 6A). Whole blood from adrenalectomized SHR caused a decrease in pressure compared with that from SHR. Dexamethasone-treatment also induced a rise in pressure of whole blood, but not plasma or plasma with erythrocytes (Figure 6B). The increased pressure due to dexamethasone-treatment was reduced by cytochalasin-D. The pressure with plasma (PPL) or the additional pressure drops due to erythrocytes (PRBC–PPL) were not significantly different between WKY and SHR blood (Figure 6C). In contrast, the additional pressure drop induced by leukocytes (PWB–PRBC) were significantly different in WKY and SHR.

The hematocrit of Wistar was significantly lower than that of all other groups. Although SHR as donor animals had a higher leukocyte count than WKY, there was no difference in leukocyte counts between other animal groups (Figure 7).

**MABP After Injection of SHR Blood**

Injection of whole blood from SHR, but not WKY, significantly increased MABP of Wistar (Figure 8A). The increase in MABP by SHR blood was not present when leukocytes were removed from the blood. Cytochalasin-D significantly reduced the ability of SHR blood to raise MABP (from 1 to 15 minutes after blood injection; Figure 8A). There was no
significant difference in MABP between WKY blood with and without cytochalasin-D.

**MABP After Blood Exchange Between WKY and SHR**

Blood exchange between WKY and SHR (50 mL) significantly increased MABP of WKY and modestly reduced MABP in SHR. Blood exchange between WKY gave no significant shift (Figure 8B and 8C).

**Discussion**

SHR have a higher fraction of circulating leukocytes with pseudopods as well as a reversal of their shear response. This leads to a rise of the resistance in a resting hemodynamically isolated skeletal muscle microcirculation, which could be eliminated by adrenalectomy or cytochalasin-D treatment. The current evidence suggests that the enhanced flow resistance caused by the reversal of leukocyte shear response due to glucocorticoids may be associated with an elevated capillary flow resistance, contributing to a rise in peripheral resistance in SHR.

Although the number of leukocytes in the circulation is much smaller than that of erythrocytes, leukocytes have a
The reduction of leukocyte velocity due to pseudopod formation in association with glucocorticoids may cause a substantial increase in microvascular resistance. Even cause plugging in capillaries. SHR and DX-Wistar leukocytes, about one-third of which have pseudopods (Figure 2), had longer relative transit times than WKY or Wistar in identical capillary networks (Figure 4A), indicating a reduced velocity in the presence of SHR leukocytes with pseudopods. Although leukocytes without pseudopods rarely obstructed capillaries, leukocytes with pseudopods during reduced flow produced more frequent capillary plugging. The incidence of capillary plugging in DX-Wistar was higher than that in controls under reduced blood flow velocity (Figure 4B through 4D). Because plugging is largely dependent on the size of the leukocytes, neutrophils and monocytes might be more involved than lymphocytes. The motion of leukocytes in capillaries may also be influenced by the endothelial glyocalyx.

What mechanism may raise the count of circulating leukocytes with pseudopods in the SHR? Although there is no significant difference in circulating levels of corticosteroids between WKY and SHR, glucocorticoid receptors have higher density and sensitivity in SHR than WKY, suggesting that the abnormal response to glucocorticoids is a requirement for sustained elevation of blood pressure in SHR. Fluid shear stress serves to inactivate leukocytes by down-regulation of membrane CD18 molecules and retraction of pseudopods. The latter event is a requirement to keep circulating leukocytes in a spherical shape without pseudopods. Impairment of the shear response has a profound effect on dynamics of circulating leukocytes. Our results indicate that the shear response of leukocytes is reversed in the presence of glucocorticoids: fluid shear induces pseudopod projection rather than retraction (Figure 1).

Glucocorticoids reduce leukocyte activation and leukocyte-endothelium interaction by reduction of adhesion molecule expression, inhibiting leukocyte emigration in post-capillary venules. In the presence of fluid shear, however, glucocorticoids may cause dissociation between reduced expression of adhesion molecules and enhanced pseudopod formation. Excess levels (in DX-Wistar) of, or excess response (in SHR) to glucocorticoids make leukocytes less adhesive but cause them to project more pseudopods, leading to the elevated fraction of leukocytes with pseudopods in the circulation (Figure 2). In SHR, both phenomena were prevented by adrenalectomy. The elevated blood pressure may not stimulate pseudopod formation in SHR because reduction of MABP by beta-blocker did not cause any change in fraction of leukocyte with pseudopods (Figure 2C). Although the reversal of the shear response is rapid in vitro, longer periods are required to reverse the response in vivo (Figure 2D). Because glucocorticoids have a number of effects in vivo, multiple factors may be associated with the difference between in vitro and in vivo.
in the microchannel flow array analyzer in vitro (Figure 3). After dexamethasone-treatment, shear application caused significant increases in blood passage time, in the fraction of cells with pseudopods, and in the frequency with which leukocytes are plugging microchannels (Figure 3C). In this device the microchannel resistance is directly related to the cell passage time. EDTA, which suppresses pseudopod formation in leukocytes,9 inhibited all these effects by dexamethasone. Therefore, pseudopod formation in leukocytes due to glucocorticoid-mediated reversal of the shear response elevates microvascular resistance.

We then examined in the vasodilated skeletal muscle to what degree pseudopod formation contributes to the hemodynamic resistance in vivo independent of the arterial/arteriolar control (Figure 5). At a constant flow rate, there was no significant difference in additional pressure drops induced by plasma or erythrocytes between WKY and SHR. But SHR leukocytes in whole blood caused a significantly higher pressure-drop than WKY ones, similar to DX-Wistar leukocytes compared with Wistar leukocytes. Both adrenalectomy and treatment with cytochalasin-α, an agent that reduces cytoplasmic stiffness and pseudopod formation,6 eliminated the increase in additional pressure drop induced by SHR leukocytes.

Furthermore, when the microvascular resistance was examined in resting, but not vasodilated skeletal muscle of SHR, close to its normal physiological conditions, cytochalasin-α treatment caused a significant reduction of perfusion pressure of whole blood of SHR, but not of Wistar (Figure 6D). These collective data then support our hypothesis that, in addition to the elevated number of leukocyte count (Figure 7), an elevated fraction of circulating leukocytes with pseudopods enhances peripheral hemodynamic resistance in SHR.

In addition, adrenalectomy in SHR serves to recover the shear response of leukocytes and reduce both MABP and pseudopod formation in circulating leukocytes (Figures 1 and 2). Moreover, injection of blood aliquots derived from SHR into normal animals caused a significant rise in MABP, whereas neither blood aliquots from WKY nor SHR blood without leukocytes would cause a blood pressure elevation (Figure 8A). The increase in blood pressure by a SHR blood aliquot was inhibited by cytochalasin-α, although cytochalasin-α had no effect when WKY blood was injected.

Although the mechanisms for glucocorticoid-induced hypertension are still uncertain, peroxisome proliferator-activated receptor-α may be involved.32 Blood pressure depends on several factors, including blood volume, peripheral vascular resistance, cardiac output, and blood viscosity. Among them, excess levels of glucocorticoids induce hypertension in humans and rats due to a rise in cardiac output and peripheral resistance.33,34 A rise in total peripheral resistance in SHR results mainly from the enhanced resistance in the terminal arterioles.35 Previously this was attributed exclusively to elevated arteriolar tone caused by increased reactivity to vasoconstrictors, decreased response to vasodilators, and rarefaction.1–3 Although vascular control might be one major factor responsible for an increase in blood pressure in SHR,
we now demonstrate that the elevated hemodynamic resistance due to glucocorticoid-induced pseudopod formation of circulating leukocytes, and its impact on the apparent viscosity of the erythrocyte in single file capillaries, might be another contributor in this hypertensive animal model. Interestingly, calcium channel blockers, cGMP/nitric oxide, and cAMP enhance the leukocyte shear response and/or suppress the impairment of shear response, all of which are known to be vasodilators. These may be physiologically a favorable coincidence, but it makes it difficult to examine inhibition of glucocorticoid-induced reversal of shear response of leukocytes without influencing vascular tone. In this study, we used cytochalasin-D at a dose without detectable effect on vascular tone but still prevention of pseudopod formation. There was no significant difference between WKY blood and WKY blood with cytochalasin-D (Figures 6D and 8A) although further study may be required using other microfilament blockers. In addition, blood exchange between WKY and SHR caused a significant increase in MABP in WKY (Figure 8B), which is consistent with previous reports, suggesting that pseudopod formation in SHR is in part responsible for the development of hypertension.

Because glucocorticoids are used as antiinflammatory agents, they were expected to be effective in myocardial infarction or cerebral ischemia due to inhibition of leukocyte-endothelial cell interaction. However, several trials have failed to confirm any beneficial effect. Multidose treatment with glucocorticoids even enhanced infarct volume during myocardial ischemia. This may be partly associated with enhanced capillary leukostasis by glucocorticoids in microvascular regions with reduced blood flow, such as penumbra around cerebral infarct areas. The same mechanism may be involved in several organ injuries in SHR.

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References


Figure 8. A, MABP in Wistar rats after injection of whole blood from WKY without (WKY, ○) or with (WKY cyD, △) 0.1 μmol/L cytochalasin-D, or SHR without (SHR, ●) or with (SHR cyD, ▲) cytochalasin-D, and blood without leukocytes from WKY (WKY wo WBC, ○) or SHR (SHR wo WBC, ★). Average MABP for 5 minutes before blood injection in each was set to 0 mm Hg. In SHR, values between 1 and 30 minutes are significantly higher than before injection (P≤0.0330), n=4 animals/group. *P=0.0237 vs SHR cyD. B and C, MABP after blood exchange between WKY and WKY (B), or WKY (○) and SHR (●) (C), n=6 (3 pairs) (B), or 5 (C) in each group. **P=0.0074 vs MABP at time 0 in WKY.
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Supplemental Methods

Since even mild levels of centrifugation interfere with the shear response (1), leukocyte separation by centrifugation was avoided in all the experiments.

**Animals:** After general anesthesia (sodium-pentobarbital, 50 mg/kg), the femoral veins and arteries of mature male Wistar rats (Wistar, n=100), SHRs (n=50), Wistar Kyoto rats (WKYs, n=29), adrenalectomized Wistar rats (n=4), adrenalectomized SHRs (n=7), and adrenalectomized WKYs (n=4) (290-390 g, Charles River Laboratories, Wilmington, M.A.) were cannulated. Mean blood pressure was measured in the femoral artery (MABP). Adrenalectomized animals were purchased from the breeder and received 0.9% saline in the drinking water before the experiments (1 week). The experimental protocol was approved by the University of California San Diego Animal Subjects Committee.

**Shear response of rat leukocytes in suspension in a cone-and-plate device:** Arterial blood with ammonium heparin (30 U/ml) was collected (n=4 in each animal group) and treated with or without dexamethasone (Sigma Chemical Corporation, St. Louis, MO) 30-min after blood collection. Fifty minutes after blood collection, whole blood (0.3 ml) was sheared in a cone-and-plate device at 5.0 dyn/cm² for 10 min (2), and immediately fixed with 2 % glutaraldehyde. Unsheared samples were fixed at the same time. Stained with 0.02 % crystal violet, the fraction of leukocytes with pseudopods was counted by microscopy in a blinded manner. For microphotography, erythrocytes were removed by FACS Lysing solution (Becton Dickinson), which after fixation does not affect the leukocyte shapes.
Shear response of SHR leukocytes after treatment with a beta-blocker: MABP and fraction of leukocytes with pseudopods in arterial blood were examined in SHR 1-hour after treatment with 1 mg propranolol hydrochloride (Inderal, Sumitomo, Osaka, Japan), SHR after treatment with propranolol hydrochloride (1 mg/12 hours) for 7 days, and control SHR (n=4 in each group).

Shear response of leukocytes in vivo after DX-treatment: In all groups FMLP ($10^{-9}$ mol/kg) was intravenously injected at time “0”. The fraction of leukocytes with pseudopods in arterial blood was examined in Wistar treated with dexamethasone (0.5 mg/kg, i.v.) at time “0”, Wistar treated with dexamethasone for 7 days (0.5 mg/kg/day, i.m.), and control Wistar (n=4 in each group). Arterial blood (0.1 ml) was collected from the femoral artery every 15 min from time “0” to “60” min, and immediately fixed with 2% glutaraldehyde, and the fraction of leukocytes with pseudopods was counted with microscopy in a blinded manner.

Leukocytes kinetics in the rat microcirculation: The ileocecal portion of the rat mesentery was exteriorized and superfused with a Krebs-Henseleit bicarbonate-buffered solution saturated with a 95% N$_2$ and 5% CO$_2$ mixture (36.5°C, pH 7.4). The mesenteric microcirculation was visualized through a digital intravital fluorescence microscope with silicone intensified target camera (SIT Model 66, Dage-MTI, Inc., Michigan City, Indiana) (1-3). Carboxyfluorescein succinimidyl ester (1.0 mg/kg, CFSE; Molecular Probes) served as label for circulating leukocytes.

Relative transit in mesenteric microcirculation Relative transit time (ratio of leukocytes and erythrocytes transit time) of CFSE-labeled leukocytes from WKYs and SHRs, or normotensive Wistar rats and Wistar rats treated with 0.5 mg/kg dexamethasone for 7
days (DX-Wistar) (n=3 in each) was determined. Dexamethasone (0.5 mg/kg in saline) was intramuscularly injected into the animal every day for 7 days. The measurements were carried out in the same capillary network of recipient rats (n=6) under identical MABP (4). Since transit time is a measurement of the flow rates through the capillary network, the inverse of the transit time serves as measure for microvascular resistance. The cell membrane of erythrocytes from recipient rats was labeled with the fluorescent dye PKH-26 (Sigma) and injected into the recipient rats (0.5 ml) (5). CFSE-labeled leukocytes in whole blood from either WKY or SHR (or normotensive Wistar or DX-Wistar) were observed for 10 min after a first intravenous injection. When no more labeled cells were encountered, a second aliquot of CFSE-labeled leukocytes in whole blood was injected. The order of blood sample injection was kept at random. The transit time of 50 PKH-26-labeled erythrocytes of the recipient rat and 30 leukocytes of each donor rat through the same capillary network was measured after each injection.

**In vivo leukocyte kinetics in capillaries during reduced blood flow** The flow velocity in mesenteric capillaries in Wistar (n=5) or DX-Wistar (n=5) was reduced to 0.2-1.5 mm/sec by partial occlusion of the celiac artery using an extravascular balloon (Medtronic PS Medical, Mentor Corporation, Goleta, CA). PKH-26-labeled erythrocytes were used to measure flow velocity with frame by frame analysis (5). The number of plugging leukocytes per capillary in each observation field was determined for 5 min. “Plugging leukocytes” were defined as cells that obstructed a capillary for at least 30 sec.

**Measurement of pressure-flow relation in resting hemodynamically isolated rat gracilis muscle:** The left gracilis muscle of normotensive Wistar (n=23) was hemodynamically isolated from the central circulation using microsurgical techniques (6-
9). After hemodynamic isolation of the muscle by ligation of communicating vessels, the feeder (femoral artery) was connected to a precise syringe pump (Pump 22, Multiple syringe pump, Harvard Apparatus, Holliston, MA) and the draining vein (femoral vein) was held at 0 mmHg throughout the experiments. The surgical isolation of the muscle required approximately 60 to 90 min, and a set of experiments was completed within 4 hours after the recipient animal was anesthetized. A buffer containing Plasma-Lyte (Baxter Health Care, Deerfield, IL) with 5% bovine serum albumin (Sigma), 10 U/ml ammonium heparin, and 0.015% papaverine hydrochloride (Bedford Laboratories, Bedford, OH), was perfused for 20 min to fully dilate the microvessels (9).

Blood with 30 U/ml ammonium heparin was collected from either normotensive Wistar, DX-Wistar, SHR, WKY (n=5 animals in each case), or adrenalectomized SHR (n=3). The blood was divided into 4 groups (each with 0.015% papaverine hydrochloride): 1. plasma, 2. plasma + erythrocytes, 3. whole blood, and 4. whole blood + 0.1 µM cytochalasin D (Sigma) to prevent pseudopod formation (10). Plasma was obtained from blood after centrifugation at 900 g for 10 min. Erythrocytes were obtained from the bottom part of mixed solution with blood and Histopaque-1077 (Sigma) after 1 g sedimentation for 30 min. In the “plasma + erythrocytes” group, the hematocrit was adjusted to the same values as that of donor animal blood. Cytochalasin-D was applied to the blood samples for 15 min immediately after the blood collection. The steady-state pressure-flow relationship for each blood sample was measured by stepping the input arterial flow rate in 8-10 steps up and down from 0 to ~350 µl/min in intervals of 15-20 s.
At a constant flow rate, “additional pressure drops” were determined from these curves to describe the relative effects of erythrocytes and leukocytes on the pressure-flow relationships. The additional pressure drop for erythrocytes was determined by subtraction of the arterial pressure (at constant flow rate of 150 µl/min/g) during perfusion with red cells (PRBC) from the arterial pressure for plasma only (PPL). In a similar fashion the additional pressure drop for leukocytes (PWB) was determined by subtraction of the arterial pressure for whole blood (erythrocytes and leukocytes) (PWB) from the arterial pressure for erythrocytes only (PRBC) (see Fig 5A).

In another set of experiments, the gracilis muscle of SHRs (n=10) was also hemodynamically isolated from the central circulation in a resting condition, but without vasodilation. The steady-state pressure-flow relationship for plasma, whole blood, and whole blood + cytochalasin-D of Wistar or SHR (n=5 animals in each) was measured without papaverine hydrochloride-treatment.

At the end of each set of experiments, 0.02 % crystal violet was infused to mark and weigh the perfused muscle tissue. In each animal the number of circulating leukocytes was counted (Unopette, Becton Dickinson, Franklin Lakes, NJ).

**Injection of SHR blood into normal rats** Whole blood with or without 0.1 µM cytochalasin-D (to prevent pseudopod formation) from SHR (n=6) or WKY (n=6) was injected into normal recipient Wistar (4.5 ml/kg, n=24), while MABP was measured. In selected blood samples leukocytes were removed after centrifugation (at 300 g for 5 min). Cytochalasin-D was applied to the blood samples for 15 min immediately after the blood collection.
MABP after blood exchange between WKY and SHR Between WKY and SHR (n=5 in each group), or WKY and WKY (n=6), 10 ml of arterial blood was exchanged every 5 min for 5 times, and MABP was measured 5 min after each blood exchange.

Leukocyte passage through an in vitro microchannel flow array: Due to the strong tendency for rat blood to coagulate, human blood was used in this study. Human blood with 30 U/ml ammonium heparin from volunteers was diluted in physiological saline with 1mM MgSO4 (1:1), and divided into 5 groups; unsheared and sheared control samples, 1 µM dexamethasone-treated blood without and with shear application, and dexamethasone-treated blood with 5 mM EDTA (Sigma) with shear exposure.

One-minute after blood collection, the sheared groups were exposed to a shear stress of 5.0 dyn/cm² for 3 or 13 min in a cone-and-plate device. Four-, 14-, and 19-min after blood collection, the passage time of 0.1 ml blood through the Microchannel Flow Analyzer (Kowa Co. Ltd.) was determined (11). The array of identical microchannels (equivalent diameter 5 µm, length 20 µm, 3100 parallel channels) was examined under constant suction (negative pressure of -15, -20, and -25 cmH2O). Light microscopic images of the blood passage through the microchannels were recorded on videotapes with a charge-coupled–device camera (HV-D28S, Hitachi, Tokyo) for off-line analysis. Fractions of occluded microchannels due to leukocyte plugging and leukocytes with pseudopods were evaluated 14-min after blood collection.

Statistics: All values are shown as mean ± standard deviation. Differences between groups were analyzed by ANOVA and Fischer's protected least significant difference test. p<0.05 was considered significant.
References for Supplemental Methods


