Spontaneously Hypertensive Versus Control Rat Aorta Response to Neutrophil-Derived Factors

Dorothy Nigro, Paulina Sannomiya, Maria Helena Catelli de Carvalho, Regina Scivoletto, Zuleica Bruno Fortes

Abstract We designed experiments to study the interaction of activated rat peritoneal neutrophils with aortas from spontaneously hypertensive rats (SHR) compared with those from normotensive rats. In aortic rings precontracted with phenylephrine, neutrophils obtained from normotensive rats caused a cell number-dependent relaxation of normotensive rat aorta with or without endothelium, whereas relaxation followed by contraction was observed in SHR aorta with endothelium. In SHR aortic rings denuded of endothelium, neutrophils did not induce contraction. The relaxation might be due to a factor indistinguishable from nitric oxide. The contraction might be due to prostaglandin H₂ because it was blocked by indomethacin, a cyclooxygenase inhibitor, and ridogrel, a thromboxane A₂ synthetase inhibitor/thromboxane A₂-prostaglandin H₂ antagonist, but not by superoxide dismutase, a superoxide anion scavenger, or dazoxiben, a thromboxane A₂ synthetase inhibitor. SHR neutrophils caused a cell number-dependent relaxation of normotensive rat aorta with or without endothelium, whereas relaxation followed by contraction was observed in SHR aorta with endothelium. In SHR aortic rings denuded of endothelium, neutrophils did not induce contraction. The relaxation might be due to a factor indistinguishable from nitric oxide. The contraction seems to be due to superoxide anion because it was inhibitable by indomethacin and superoxide dismutase but not by dazoxiben and ridogrel. Equivalent amounts of superoxide anion were produced by unstimulated and phorbol myristate acetate-stimulated neutrophils obtained from either SHR or normotensive rats. Therefore, increased production of this anion could not explain the contraction observed in hypertensive aortas. In conclusion, our data might indicate a role for neutrophils in the elevated peripheral vascular resistance in hypertension because an endothelium-dependent contraction was induced by neutrophils in vessels of hypertensive rats. (Hypertension. 1994;24:728-733.)

Key Words • neutrophils • hypertension, spontaneous • rats, inbred SHR • endothelium-derived relaxing factors

The interaction of polymorphonuclear leukocytes (PMNs) and blood vessels may be important in the control of vascular tone because activated neutrophils release a variety of vasoactive factors. Rat peritoneal PMNs continuously release a soluble factor that is inactivated by superoxide anions and that induce vascular smooth muscle relaxation via the elevation of cyclic GMP levels. Therefore, this neutrophil-derived relaxing factor has a pharmacological profile similar to that of endothelium-derived relaxing factor. On the other hand, activated neutrophils generate oxygen-derived metabolites, superoxide anion and hydrogen peroxide, which may indirectly affect vascular tone by inactivating endothelium-derived relaxing factor and diminishing prostacyclin (PGI₂) release, respectively.

Although leukocytes may be involved in the control of vascular tone, little is known about the role of these cells in hypertension. Schmid-Schönbein et al speculate that leukocytes contribute to the pathogenesis of vascular changes and subsequent organ injury in hypertension. Vascular reactivity has been shown to be altered in experimental models of hypertension and in humans. An impaired response to acetylcholine, an endothelium-dependent vasodilator, has been demonstrated. Since cyclooxygenase inhibitors correct the impaired response, the release of an arachidonic acid metabolite via this enzyme has been implicated in the response. Prostaglandin H₂ (PGH₂) is a candidate for this endothelium-derived factor in spontaneously hypertensive rats (SHR).

To study the interaction of PMNs obtained from SHR or normotensive rats with isolated vessels of SHR and normotensive rats and the factor or factors liberated as a consequence of this interaction, we investigated the response of isolated aortas from SHR and normotensive rats to PMN suspensions.

Methods

A total of 250 male SHR and 260 normotensive Wistar rats (200 to 250 g) were used. All rats were derived from breeding stock maintained at our institute. All animal procedures were in accordance with institutional guidelines.

PMN Preparations

Leukocyte suspensions were prepared according to Rimele et al with slight modifications. Briefly, leukocytes were obtained from SHR or Wistar rats 4 hours after intraperitoneal injection of 20 mL of a 1% oyster glycogen (type II) solution. The animals were anesthetized with ether and the cells collected by rinsing the abdominal cavity with an adequate volume of physiological saline containing 1 IU/mL heparin. Cells were centrifuged at 450g for 7 minutes and the contaminating erythrocytes lysed by the addition of hypotonic saline. The cells were then washed twice in Hanks’ balanced salt solution.
Cells were counted with an automatic cell counter (CELM), and at least 95% of all leukocytes were PMNs. Their viability was assessed by the trypan blue exclusion method and was more than 95%. PMNs (10×10^7/mL) were suspended in HBSS containing 0.1% bovine serum albumin until they were suspended in the organ chamber (30 to 60 minutes). For all experiments, cells from five to six rats were pooled, with each rat yielding 2×10^5 cells. PMNs obtained from normotensive rats were assayed in aortic preparations isolated from SHR and normotensive rats. PMNs obtained from SHR were also assayed in aortic preparations isolated from SHR and normotensive rats.

Aortic Ring Preparations

The thoracic aortas were removed from male SHR or Wistar rats previously anesthetized with chloral hydrate (300 mg/kg IP supplemented with 30 mg/kg IV as needed). After removal of loose connective tissue, two transverse rings approximately 4 mm in length were cut and mounted at the optimal length for isometric tension recording in a 10-mL organ chamber, according to Furchgott and Zawadzki. While one ring served as a control, the endothelium was mechanically removed from the other by gently rubbing the luminal surface with a small cylindrical piece of artificial sponge attached to a thread, thus permitting insertion into the lumen. Two L-shaped stainless steel wire hooks were used to mount each ring in the organ chamber containing Krebs-Henseleit solution of the following composition (mmol/L): NaCl 113, KCl 4.7, CaCl₂ 2.5, NaHCO₃ 25, MgSO₄ 1.1, ascorbic acid 0.11, EDTA 0.03, and glucose 11. The bathing fluid, maintained at 37°C, was saturated with a gas mixture of 95% O₂ and 5% CO₂. The preparations were allowed to equilibrate for at least 1 hour under a resting tension of 1.5 g, which was maintained throughout the experiment. Developed tension was detected with an F-60 microdisplacement myograph and recorded on an MK IV polygraph (Narco BioSystems). In all experiments, the aortas used in the organ chamber experiments were not those from the animals treated with the oyster glycogen (PMN donors).

Superoxide Anion Production Assay

Superoxide anion was measured as the superoxide dismutase-inhibitable reduction of cytochrome c. The reaction mixture contained 3×10⁻⁴ unstimulated or stimulated PMNs in HBSS and 100 mmol cytochrome c. To control samples, 90 IU superoxide dismutase was added to determine the level of nonspecific cytochrome c reduction. The mixtures were incubated for 20 minutes at 37°C, and the absorbance of the supernatant fluid was determined spectrophotometrically at 550 nm. Phorbol myristate acetate (PMA) was used for cell stimulation. PMA was dissolved in dimethyl sulfoxide and stored at −20°C. For use, the solution was diluted in HBSS. PMNs obtained from SHR and normotensive rats were compared in their capacity to generate superoxide anions.

Study Protocols

Parallel sets of aortic rings with and without endothelium were stimulated with phenylephrine (3×10⁻⁷ and 10⁻⁶ mol/L in rings with and without endothelium, respectively). Once a stable contraction was achieved, PMNs were added to the organ chamber at 10-minute intervals up to 8×10⁷ cells. To determine whether eicosanoids participate in the PMN-mediated vascular effects, we added indomethacin (10⁻⁴ mol/L) to inhibit cyclooxygenase, dazoxiben (10⁻⁵ mol/L) to block TXA₂/PGI₂ receptors, and ridogrel (5×10⁻⁵ mol/L) to block TXA₂ synthesizes, or ridogrel (5×10⁻⁵ mol/L) to block TXA₂/PGI₂ receptors to the organ bath 30 minutes (for indomethacin) or 1 hour (for dazoxiben and ridogrel) before the beginning of the addition of PMNs and maintained them throughout. To clarify whether the cyclooxygenase inhibitor acted on PMNs or vascular rings, we pretreated PMN suspensions or vascular preparations with indomethacin. After preincubation for 30 minutes, indomethacin was washed out and the cells or aortic rings were tested. To study the participation of the superoxide anion on the PMN-mediated vascular effects, we added superoxide dismutase (100 IU/mL) to the organ bath 5 minutes before the beginning of the addition of PMNs and maintained it throughout.

To verify the effect of PMN suspensions (2×10⁷ to 8×10⁷ cells) on baseline tension, we also tested phenylephrine-unstimulated aortic rings with and without endothelium.

Drugs

Phenylephrine, superoxide dismutase, cytochrome c, PMA, dimethyl sulfoxide, and oyster glycogen (type II) were obtained from Sigma Chemical Co. Indomethacin was kindly supplied by Merck Sharp & Dohme, dazoxiben by Pfizer Co, and ridogrel by Janssen Pharmaceutica.

Phenylephrine, dazoxiben, and ridogrel were dissolved in Krebs-Henseleit solution, indomethacin was dissolved in Tris buffer (pH 8.0), and oyster glycogen was prepared in physiological saline (154 mmol/L NaCl solution). Drug concentrations are expressed as the final molar concentration in the organ chamber, except for superoxide dismutase, which is expressed as units per milliliter in the organ chamber.

Statistical Analysis

Data are given as the mean±SEM. One-way ANOVA for repeated measurements was used. To test differences among means, Scheffé’s test was used. The minimum acceptable level of significance was set at a value of P<.05.

Results

PMNs From Normotensive Rats and Aortic Reactivity

The addition of PMNs (2×10⁷ to 8×10⁷ cells per organ chamber) from normotensive rats did not affect the baseline tension in either endothelium-intact or endothelium-denuded aortic rings isolated from normotensive rats or SHR. On the other hand, the addition of PMNs to phenylephrine-precontracted rings of normotensive rats caused a cell number–dependent relaxation that was maximal at 8×10⁷ cells in both endothelium-intact and endothelium-denuded preparations (Fig 1A). The addition of PMNs to phenylephrine-precontracted rings of SHR caused relaxation at 2×10⁷ and 4×10⁷ cells followed by contraction at 6×10⁷ and 8×10⁷ cells in endothelium-intact preparations. In endothelium-denuded preparations, greater relaxation to PMN addition was observed compared with endothelium-intact preparations (Fig 1A).

PMNs From SHR and Aortic Reactivity

The addition of PMNs (2×10⁷ to 8×10⁷ cells per organ chamber) from SHR did not affect the baseline tension in either endothelium-intact or endothelium-denuded aortic rings isolated from normotensive rats or SHR. On the other hand, the addition of PMNs to phenylephrine-precontracted rings of normotensive rats caused a cell number–dependent relaxation that was maximal at 8×10⁷ cells in both endothelium-intact and endothelium-denuded preparations (Fig 1B). The addition of PMNs to phenylephrine-precontracted rings of SHR caused relaxation at 2×10⁷ and 4×10⁷ cells followed by contraction at 6×10⁷ and 8×10⁷ cells in endothelium-intact preparations. In endothelium-denuded preparations, greater relaxation to PMN addition was observed compared with endothelium-intact preparations (Fig 1B).
Figure 1. Line graphs show relaxation induced by polymorphonuclear leukocytes (PMNs) in phenylephrine-precontracted aorta isolated from normotensive rats (A, circles) or spontaneously hypertensive rats (B, triangles). Closed symbols represent aortas with endothelium (+E); open symbols, aortas without endothelium (−E). The abscissa shows PMN number ×10⁷ cells per 10-mL organ chamber. Data are mean±SEM of five to eight experiments. *P<.05 relative to preparations without endothelium.

Superoxide Anion Production by PMNs

We assayed superoxide anion production by PMNs to verify whether an increased release of this anion occurred and could explain the contraction observed in endothelium-intact SHR aortas. However, equivalent amounts of superoxide were produced by PMA-stimulated PMNs obtained from SHR and normotensive rats. In addition, there were no differences between unstimulated PMNs from SHR and normotensive rats with respect to superoxide anion production. The Table summarizes the results.

PMNs From Nonhypertensive Rats and Aortic Reactivity of SHR

Effect of Indomethacin

To determine the chemical mediator that caused the PMN-induced contraction in SHR vessels, we added an arachidonate cyclooxygenase inhibitor (indomethacin, 10⁻⁵ mol/L) to the organ chamber 30 minutes before the addition of PMN suspension. This agent abolished the contraction induced by PMNs obtained from normotensive rats and increased the response of SHR preparations. The Table summarizes the results.

Production of Superoxide Anion by Polymorphonuclear Leukocytes Stimulated With Phorbol Myristate Acetate

<table>
<thead>
<tr>
<th>Cell Donor</th>
<th>Resting (nmol/3×10⁷ cells)/20 min</th>
<th>PMA-Stimulated (100 ng) (nmol/3×10⁷ cells)/20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>0.12±0.03</td>
<td>4.69±0.73</td>
</tr>
<tr>
<td>Normotensive rats</td>
<td>0.09±0.03</td>
<td>3.52±0.76</td>
</tr>
</tbody>
</table>

PMA indicates phorbol myristate acetate; SHR, spontaneously hypertensive rats. Cells obtained from SHR or normotensive rats were suspended in Hanks’ balanced salt solution in the absence or presence of phorbol myristate acetate for testing. In each group, values are mean±SEM of 10 separate experiments done in duplicate.
**Effect of Superoxide Dismutase**

To determine the role of superoxide anion on the contraction induced by PMNs from normotensive rats in SHR vessels, we added superoxide dismutase (100 IU/mL) to the organ chamber 5 minutes before the addition of PMN suspensions. This treatment was not capable of abolishing the contraction induced by PMNs from normotensive rats in endothelium-intact SHR preparations. Rubbed preparations did not exhibit a contraction and were not altered by superoxide dismutase treatment (Fig 2).

**Effect of Dazoxiben**

To verify whether TXA$_2$ was involved in the contraction induced by PMNs from normotensive rats in SHR vessels, we used dazoxiben (10$^{-4}$ mol/L) to inhibit TXA$_2$ synthetase. This agent did not abolish the contraction induced by PMNs from normotensive rats in endothelium-intact SHR preparations. Rubbed preparations were not altered by dazoxiben treatment (Fig 2).

**Effect of Ridogrel**

To verify whether TXA$_2$ and/or PGH$_2$ were involved in the contraction induced by PMNs from normotensive rats in SHR vessels, we used ridogrel (5$\times$10$^{-5}$ mol/L), a TXA$_2$ synthetase inhibitor and TXA$_2$/PGH$_2$ receptor antagonist. This agent abolished the contraction induced by PMNs from normotensive rats in endothelium-intact SHR preparations. After ridogrel treatment, the response induced by PMN suspensions in endothelium-denuded preparations was not different from that obtained in endothelium-intact preparations (Fig 2).

**PMNs From SHR and Aortic Reactivity of SHR**

**Effect of Indomethacin**

To determine the chemical mediator that caused the PMN-induced contraction in SHR vessels, we added an arachidonate cyclooxygenase inhibitor (indomethacin, 10$^{-5}$ mol/L) to the organ chamber 30 minutes before the addition of PMN suspension. This agent abolished the contraction induced by PMNs obtained from SHR in endothelium-intact SHR preparations. The response induced by PMN suspensions in endothelium-denuded preparations was not different from that obtained in endothelium-intact preparations (Fig 3). Aortic rings obtained from normotensive rats did not develop contraction to the addition of PMNs from SHR; therefore, indomethacin treatment did not alter the response pattern (data not shown).

To clarify whether the cyclooxygenase inhibitor acted on PMNs or vascular rings, we pretreated PMN suspensions or vascular preparations with indomethacin. After preincubation of PMNs for 30 minutes, the cells were washed and resuspended in saline and then added to the ring preparations. PMN-induced contraction was significantly attenuated (by 60%). After preincubation of vascular rings with indomethacin for 30 minutes, this agent was washed out. PMN addition to aortic rings precontracted with phenylephrine induced an attenuated contraction (data not shown). Therefore, indomethacin must remain in contact throughout to abolish completely the contraction induced by PMN suspension. The concentration used was sufficient for complete inhibition of the production of cyclooxygenase metabolites by PMNs or vascular preparations in preliminary experiments.

**Effect of Superoxide Dismutase**

To determine the role of superoxide anion on the contraction induced by PMNs from SHR in SHR vessels, we added superoxide dismutase (100 IU/mL) to the organ chamber 5 minutes before the addition of PMN suspensions. This treatment was not capable of abolishing the contraction induced by PMNs from normotensive rats in endothelium-intact SHR preparations. Rubbed preparations did not exhibit a contraction and were not altered by superoxide dismutase treatment (Fig 2).

**Effect of Dazoxiben**

To verify whether TXA$_2$ was involved in the contraction induced by PMNs from SHR in SHR vessels, we used dazoxiben (10$^{-4}$ mol/L) to inhibit TXA$_2$ synthetase. This agent did not abolish the contraction induced by PMNs from SHR in endothelium-intact preparations. Rubbed preparations were not altered by dazoxiben treatment (Fig 2).

**Effect of Ridogrel**

To verify whether TXA$_2$ and/or PGH$_2$ were involved in the contraction induced by PMNs from SHR in SHR vessels, we used ridogrel (5$\times$10$^{-5}$ mol/L), a TXA$_2$ synthetase inhibitor and TXA$_2$/PGH$_2$ receptor antagonist.
This agent neither abolished the contraction induced by PMNs from SHR in endothelium-intact SHR preparations nor altered the response induced by PMNs from SHR in denuded preparations (Fig 3).

**Discussion**

In the present study, we demonstrated that activated PMNs from SHR caused cell number–dependent relaxation in endothelium-intact aortic rings from normotensive rats, whereas a biphasic curve (relaxation at lower and contraction at higher concentrations) was obtained in endothelium-intact SHR preparations. Therefore, the endothelium and smooth muscle of SHR respond to PMNs differently than the endothelium and smooth muscle of normotensive rats. A recent study suggests that PMNs release a vasodilator substance that has a pharmacological profile indistinguishable from nitric oxide. Therefore, the relaxation induced by these cells in SHR and normotensive aortas might be caused by the production of nitric oxide. Although an increased production of superoxide anion by PMNs could be responsible for the contraction induced by PMNs at higher concentrations, this is not likely in the present study because equivalent amounts of superoxide anion were generated from PMNs obtained from either SHR or normotensive rats. The contraction induced by PMNs from SHR in SHR aortas was sensitive to indomethacin inhibition; therefore, a product of arachidonic acid metabolism might be involved. Since dazoxiben and ridogrel had no significant effect on the PMN-induced endothelium-dependent contraction observed in SHR aorta, TXA₂ and PGH₂ could be excluded. However, superoxide dismutase treatment abolished the contraction. Thus, cell-cell interaction of hypertensive cells with hypertensive vessels results in the production of an indomethacin- and superoxide dismutase–sensitive product, probably superoxide anion. The contraction could be explained by an increased response of SHR vessel wall to the anion. In fact, increased contraction to oxygen-derived free radicals has been demonstrated in SHR aorta by Auch-Schwelk et al.

Vascular tone is a result of a balance between vasoconstrictor and vasodilator factors. Vascular endothelium releases relaxing factors such as endothelium-derived relaxing factor/nitric oxide, endothelium-derived hyperpolarizing factor, and PGH₂ as well as contracting factors such as oxygen free radicals, PGH₂, and TXA₂ might be involved in the control of vascular tone. Previous studies have shown that PMNs can affect vascular tone by several mechanisms. Among these mechanisms, superoxide anions released from PMNs have received increasing attention as a cause of endothelium-dependent contraction to PMNs. Therefore, our data showing an endothelium-dependent contraction induced by PMNs in SHR vessels might help to explain the increased vascular tone in hypertension.

The cell-cell contact, that is, the neutrophil-vessel interaction of cells derived from normotensive rats and SHR, does not seem to be the same. In fact, PMNs from normotensive rats caused cell number–dependent relaxation in aortic rings from normotensive rats and a biphasic curve (relaxation at lower and contraction at higher concentrations) in endothelium-intact SHR preparations. However, although indomethacin abolished the contraction observed in SHR aortas, superoxide dismutase was not capable of abolishing it. These findings allow us to exclude superoxide anion release as responsible for the contraction. Since ridogrel, a TXA₂ synthetase inhibitor and TXA₂/PGH₂ receptor antagonist, abolished the contraction induced by PMNs from normotensive rats in SHR vessels and dazoxiben did not affect it, we might suggest that the cell-cell interaction of normotensive cells with hypertensive vessels results in the production of an indomethacin- and ridogrel-sensitive product, probably PGH₂.

The differences in the efficacy of superoxide dismutase and ridogrel in reducing the endothelium-dependent contraction in response to the cells showed that the interaction of the neutrophils with the vascular tissue results in a differential production of either PMNs or endothelium-derived factors. Furthermore, smooth muscle and endothelial cells of aorta exhibited different sensitivities to PMNs regardless of their origin.

Evidence accumulating in the past decade suggests that leukocytes not only serve a beneficial immunological function but are directly involved in the etiology of cardiovascular diseases. They were identified as a significant contributor to blood flow resistance under normal perfusion conditions and as having an even more dramatic role during sepsis or disease states. Producing oxygen free radicals and mediators of proteolytic tissue degradation, they may participate in the organ injury during hypertension. This might be aggravated by the increased tendency exhibited by stimulated granulocytes to adhere to the vascular endothelium and by the increase in the total leukocytes as well as in the number of spontaneously activated granulocytes observed in mature and old SHR.

Endothelial dysfunction may lead to altered responses to vasoactive agents and could in part contribute to the elevated peripheral vascular resistance observed in hypertension. The results obtained in the present study allow us to suggest that altered vascular responses to PMNs might amplify the endothelium dysfunction in this pathology. Besides the decreased response to endothelium-dependent vasodilators such as acetylcholine, ADP, and thrombin, PMNs, inducing endothelium-dependent contraction in SHR vessels, would contribute to increasing the vascular tone even more, being an additional factor in the elevation of peripheral vascular resistance in hypertension.

**Acknowledgments**

This work had the financial support of FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo). The authors wish to thank Hermes Vieira Barbeiro and Claudia Becker for technical assistance.

**References**


Spontaneously hypertensive versus control rat aorta response to neutrophil-derived factors.

D Nigro, P Sannomiya, M H de Carvalho, R Scivoletto and Z B Fortes

Hypertension. 1994;24:728-733
doi: 10.1161/01.HYP.24.6.728

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/24/6/728

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/