Vascular Endothelial Growth Factor-Mediated Endothelium-Dependent Relaxation Is Blunted in Spontaneously Hypertensive Rats

MING-HUI LIU, HONG-KUI JIN, H. STORM FLOTEN, QIN YANG, ANTHONY P. C. YIM, ANTHONY FURNARY, THOMAS F. ZIONCHECK, STUART BUNTING, and GUO-WEI HE

Cardiovascular Research, Starr Academic Center for Cardiac Surgery, Providence Heart Institute, Portland, Oregon (M.-H.L., S.F., A.F., G.-W.H.); Cardiovascular Research, Genentech, Inc. San Francisco, California (H.-K.J, T.F.Z, S.B.); and Cardiovascular Surgical Research Laboratory, Department of Surgery, The Chinese University of Hong Kong, Hong Kong SAR, China (Q.Y., A.P.C.Y., G.-W.H.)

Received June 6, 2000; accepted October 5, 2000 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The vasodilatory effect of VEGF has not been characterized in the setting of hypertension. This study investigated the in vitro vasorelaxant effects of VEGF in organ chambers in the aorta of the adult (12-week-old) spontaneously hypertensive rats (SHR), young (4-week-old) SHR without hypertension, and age-matched Wistar-Kyoto (WKY) rats compared with acetylcholine (ACh). Cumulative concentration-relaxation curves were established for VEGF (∼10⁻¹²–10⁻⁸ M) and ACh (∼10⁻¹⁰–10⁻⁸ M) in U46619 (10⁻⁸ M)-induced contraction. VEGF induced endothelium-dependent relaxation that was significantly reduced in the adult SHR compared with the age-matched WKY control (87.8 ± 2.8 versus 61.4 ± 8.6%, P = 0.01). These responses were significantly attenuated by pretreatment with N⁶-nitro-L-arginine (L-NNA, 300 μM) alone (SHR: 25.1 ± 1.9%; WKY: 21.0 ± 2.6%; P = 0.01) or indomethacin (7 μM) + L-NNA (SHR: 30.2 ± 2.1%; WKY: 35.0 ± 2.9%; P = 0.01). Further addition of oxyhemoglobin (20 μM) abolished the residual relaxation and reduced the relaxation induced by nitroglycerin. ACh induced similar responses to VEGF. In contrast, pretreatment with indomethacin alone enhanced VEGF- or ACh-induced relaxations and the effect was greater in the adult SHR than in WKY rats. In contrast to the adult SHR versus WKY rats, there were no significant differences of VEGF- or ACh-induced relaxations between young SHR and WKY rats. The results demonstrate that VEGF induces endothelium- or nitric oxide-dependent relaxation, which is blunted in the adult SHR. The mechanism of this impairment may be related to decreased release of NO although increased release of contracting factors from the dysfunctional endothelium may also be involved.

Vascular endothelial growth factor (VEGF) is a basic, heparin-binding, homodimeric glycoprotein that is specifically mitogenic for endothelial cells (Senger et al., 1983; Leung et al., 1989; Koch et al., 1994; Dvorak et al., 1995; Ferrara et al., 1995). VEGF has its high-affinity binding sites localized to the endothelium of both large and small vessels, but not to other cell types (Jakeman et al., 1992). As a major regulator of physiological and pathological angiogenesis, VEGF has been shown to promote endothelial cell proliferation and migration in vitro (Leung et al., 1989; Koch et al., 1994) and to induce a strong angiogenic response in the setting of myocardial or peripheral vascular ischemia (Banai et al., 1994; Takeshita et al., 1994; Bauters et al., 1995; Pearlman et al., 1995; Harada et al., 1996; Hariawala et al., 1996). Similarly, a single intracoronary bolus of VEGF is successful in improving blood flow in a peripheral model of vascular insufficiency and ischemia (Pu et al., 1993; Takeshita et al., 1994; Bauters et al., 1995). It has been shown, however, that systemic or intracoronary administration of VEGF results in a significant depressor response in the various species of animals, which has been attributed to the profound vasodilation (Horowitz et al., 1995; Yang et al., 1996; Lopez et al., 1997; Yang et al., 1998). VEGF-induced vascular relaxation is shown to be NO-related (Ku et al., 1993; Yang et al., 1996) and VEGF is known to regulate endothelial NO synthase expression in cell culture (Shen et al., 1999).

Despite numerous studies on the vasorelaxant effect of the coronary system, intracoronary slow release or perivascular delivery of VEGF is effective in promoting collateral development and improving myocardial blood flow in several animal models (Banai et al., 1994; Pearlman et al., 1995; Harada et al., 1996; Hariawala et al., 1996). Similarly, a single intracoronary bolus of VEGF is successful in improving blood flow in a peripheral model of vascular insufficiency and ischemia (Pu et al., 1993; Takeshita et al., 1994; Bauters et al., 1995). It has been shown, however, that systemic or intracoronary administration of VEGF results in a significant depressor response in the various species of animals, which has been attributed to the profound vasodilation (Horowitz et al., 1995; Yang et al., 1996; Lopez et al., 1997; Yang et al., 1998). VEGF-induced vascular relaxation is shown to be NO-related (Ku et al., 1993; Yang et al., 1996) and VEGF is known to regulate endothelial NO synthase expression in cell culture (Shen et al., 1999).

ABBREVIATIONS: VEGF, vascular endothelial growth factor; NO, nitric oxide; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; ACh, acetylcholine; L-NNA, N⁶-nitro-L-arginine; EDCF, endothelium-derived contracting factor; EDHF, endothelium-derived hyperpolarizing factor.
VEGF, this has never been studied in the setting of hypertension. Hypertension is known to be associated with endothelial dysfunction and impaired endothelium-dependent relaxation (Van de Voorde and Leusen, 1986; Rubanyii et al., 1993; Vanhoutte and Boulanger, 1995; Tesfainiam and Ogletree, 1995; Cardillo and Panza, 1998; Rizzoni et al., 1998; Shimokawa, 1998). However, it is unknown whether the VEGF-induced vasorelaxation is altered in hypertension. We therefore designed the present study to examine the VEGF-induced vasorelaxation in the isolated rat aorta of hypertensive SHR, prehypertensive SHR, and age-matched Wistar-Kyoto (WKY) rats with regard to the role of NO and other endothelium-derived relaxing factors. The effect of VEGF was compared with that of acetylcholine (ACh).

**Experimental Procedures**

**Animal Preparation**

Male SHR and normotensive WKY rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA) at 12 weeks (adult) and 4 weeks (young) of age. Rats were fed standard rat chow and had free access to tap water. Systemic blood pressure was measured by tail-cuff plethysmography in conscious rats.

The rats were killed by CO₂ inhalation. The thoracic aorta was excised immediately and placed into a container with oxygenated physiological solution ( Krebs') maintained at 4°C and delivered to the laboratory. All aorta preparations were tested within 6 h. The Krebs' solution had the following composition: 144 mM Na⁺, 5.9 mM K⁺, 2.5 mM Ca²⁺, 1.2 mM Mg²⁺, 128.7 mM Cl⁻, 25 mM HCO₃⁻, 1.2 mM SO₄²⁻, 1.2 mM H₂PO₄⁻, and 11 mM glucose. The solution was aerated with a gas mixture of 95% O₂ and 5% CO₂.

The investigation was in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1985). The procedures and protocols of the study were in agreement with the institutional guidelines and were approved by the Animal Experimentation Committee of the Oregon Health Sciences University.

**Organ Bath Technique**

The rat aorta was placed in a glass dish with oxygenated Krebs' solution and the surrounding connective tissue was dissected out. The vessel was cut into 3-mm-long ring segments and the number of segments taken from each animal was 8 or 10. In some aortic ring segments, the endothelium was denuded by gently rubbing the intimal surface with a thin polyethylene tube. In the remaining segments, great care was taken not to touch the inner surface of the blood vessels. We found that this technique allowed the experiment to be carried out with functionally intact endothelium, as determined by the relaxation response to ACh.

Aortic ring segments (224) were investigated in the present study. Artery ring segments were mounted on two thin parallel stainless steel wire hooks in a 25-ml glass organ bath containing Krebs' solution, maintained at 37°C and continuously bubbled with 95% O₂ and 5% CO₂. The lower wire hook was attached to a micrometer-adjustable support leg and the upper to an isometric force transducer (model PT03; Grass Instruments, Quincy, MA) to record changes in isometric force, which were amplified and recorded on a polygraph chart recorder (model 79; Grass Instruments). After a 60-min equilibration period, a normalization technique was applied to set the vascular ring segments at a pressure comparable to that at the in vivo situation. The details of this technique have been previously published (He et al., 1989a,b). Briefly, each arterial segment was stretched up in progressive steps to determine the individual length-tension curve. A computer iterative fitting program (VESTAND 2.1; Yang-Hui He, Princeton University, NJ) was used to determine the exponential curve, the pressure, and the internal diameter. When the transmural pressure on each ring reached 100 mm Hg, determined from its own length-tension curve, the stretch-up procedure was stopped and the ring was released to 90% of its internal circumference at 100 mm Hg. This degree of the passive tension was then maintained throughout the experiment. After the normalization procedure, the aortic ring segments were equilibrated for at least 60 min.

**Experimental Protocol**

A cumulative concentration-response curve to the thromboxane A₂ mimetic U46619 (10⁻¹⁰–10⁻⁶.5 M) was generated. Our preliminary study showed that U46619 at 10⁻⁸ M induced ~60 to 80% of maximal contractile responses in the rat aortic ring segments.

**Adult SHR and WKY.** For both adult SHR and WKY rats, the aortic ring segments from the same rat were divided into six groups (n = 8 in each group). There was one group of ring segments with denuded endothelium. In four groups, the aortic ring segments with intact endothelium were incubated with N²-nitro-L-arginine (L-NNA, 300 µM), indomethacin (7 µM), indomethacin (7 µM) + L-NNA (300 µM), indomethacin (7 µM), indomethacin (7 µM) + L-NNA (300 µM) + oxyhemoglobin (20 µM), respectively, for 30 min before precontraction started. In the other group, the aortic ring segments with intact endothelium was incubated with vehicle (ethanol), served as control.

**Young SHR and WKY.** For both young SHR and WKY rats, the aortic ring segments from the same rat were divided into three groups (n = 8 in each group). The segments were incubated with L-NNA (300 µM, group I) or indomethacin (7 µM, group II) for 30 min. The group III was incubated with vehicle as control.

All the ring segments were precontracted with U46619 at concentration of 10⁻⁸ M. When the contraction reached a stable plateau (about 10 min), cumulative concentration-relaxation curves to VEGF (10⁻¹²–10⁻⁸.5 M) or ACh (10⁻⁸–10⁻⁵ M) were established. If the maximal response did not reach full relaxation, 300 µM nitroglycerin was added to the organ bath to observe whether there was a further relaxation. The relaxation was expressed as percentage of reversal of the U46619-induced precontraction. Only one dose-response curve was established in each ring segment of aorta.

**Data Analysis**

The sensitivity of VEGF or ACh was expressed as the EC₅₀, the effective concentration causing 50% of maximal relaxation (Rmax). The EC₅₀ was determined from each individual concentration-relaxation curve by a sigmoid logistic curve-fitting equation: E = MA / (A¹ + K¹), where E is the response, M is Rmax, A is concentration, K is EC₅₀ of concentration, and p is the slope parameter. A computerized program was used for the curve fitting and EC₅₀ values were determined and expressed as log₁₀ M.

Statistical analysis was performed with SPSS software (SPSS, Inc., Chicago, IL). All values were expressed as mean ± S.E.M. Statistical comparisons of the percentage relaxation under different treatments were performed by two-way ANOVA (general linear model) with repeated measures, followed by post hoc Bonferroni test to detect the individual differences. Rmax was compared by one-way ANOVA followed by post hoc Bonferroni test. Differences between two matched groups were determined by paired-samples t test. P < 0.05 was considered statistically significant. The 95% confidence interval for difference was also shown when possible. n values refer to number of ring segments from separate rats.

**Materials**

Drugs used in this study and their sources were as follows: L-NNA, indomethacin, hemoglobin, ACh, and nitroglycerin (Sigma Chemical Co., St. Louis, MO) and U46619 (Cayman Chemical, Ann Arbor, MI). Stock solutions of U46619 and ACh were held frozen until required. VEGF was generously provided by Genentech, Inc. (South San Francisco, CA). All solutions were freshly prepared before daily use and protected from light.
Results

A significant increase in systolic blood pressure was observed in adult SHR versus WKY rats (166.5 ± 1.2 versus 126.0 ± 1.6 mm Hg, P = 0.001) but not in young SHR versus WKY rats (104.5 ± 2.4 versus 101.3 ± 2.2, N.S.). The internal diameters of the aortic ring segments at an equivalent transmural pressure of 100 mm Hg (D100, mm) determined from the normalization procedure were similar between adult SHR and WKY rats (2.05 ± 0.02 and 2.18 ± 0.02, N.S.) and between young SHR and WKY rats (1.82 ± 0.01 and 1.92 ± 0.01, N.S.). The equivalent transmural pressures of the aortic ring segments set at a resting diameter of 90% D100 (mm) were not different between adult SHR and WKY rats (80.3 ± 0.4 and 82.0 ± 0.3, N.S.) and between young SHR and WKY rats (83.9 ± 0.3 and 83.6 ± 0.6, N.S.). The resting forces (g) of the aortic ring segments were 4.49 ± 0.07 and 4.43 ± 0.06 in the adult SHR and WKY rats (N.S.), and 4.43 ± 0.58 and 5.15 ± 0.11 in the young SHR and WKY rats (N.S.). The U46619-induced precontraction forces (g) were 1.94 ± 0.07 and 2.19 ± 0.08 in the adult SHR and WKY rats (N.S.), and 1.23 ± 0.02 and 1.32 ± 0.06 in the young SHR and WKY rats (N.S.).

VEGF induced concentration-dependent relaxation in aortic ring segments with intact endothelium in both adult and WKY rats (Fig. 1). The relaxations induced by VEGF were significantly diminished in the adult SHR compared with age-matched WKY rats (61.4 ± 8.6 versus 87.8 ± 2.8%, P = 0.01; EC50: −9.96 ± 0.18 versus −10.16 ± 0.15 log10 M, P > 0.05). Treatment with cyclooxygenase inhibitor indomethacin produced a significant enhancement in the VEGF-induced relaxations, which was greater in adult SHR than WKY rats. In contrast, the relaxations in response to VEGF were significantly attenuated by treatment of both l-NNA alone and indomethacin + l-NNA (SHR: 25.1 ± 1.9 and 30.2 ± 2.1% versus 61.4 ± 8.6%, P = 0.01; WKY: 21.0 ± 2.6 and 35.0 ± 2.9% versus 87.8 ± 2.8%, P = 0.01). The VEGF-induced relaxation was abolished by treatment of indomethacin + l-NNA + oxyhemoglobin or by removing endothelium in aortic preparations (Fig. 1).

Similarly, ACh induced less relaxation in the aortic ring segments of the adult SHR than that of the WKY rats (52.2 ± 0.07 versus control group (two-way ANOVA).

**Fig. 1.** Mean concentration (−log10 M)-relaxation (percentage of reversal of U46619-induced precontraction) curves for VEGF in adult SHR or age-matched WKY rat aortic ring segments with intact endothelium pretreated with indomethacin (Indo, 7 μM), l-NNA (300 μM), indomethacin + l-NNA (1 + L), indomethacin + l-NNA + oxyhemoglobin (20 μM) (1 + L + Hb), or vehicle as control. Another group of aortic ring segments with endothelium denuded (E−) was also tested. n = 8 in each group. Values are expressed as mean ± standard error of the mean. *P ≤ 0.005 versus control group (two-way ANOVA).

**Fig. 2.** Mean concentration (−log10 M)-relaxation (percentage of reversal of U46619-induced precontraction) curves for acetylcholine in adult SHR or age-matched WKY rat aortic ring segments with intact endothelium pretreated with indomethacin (Indo, 7 μM), l-NNA (300 μM), or vehicle as control. Another group of aortic ring segments with endothelium denuded (E−) was also tested. n = 8 in each group. Values are expressed as mean ± standard error of the mean. *P ≤ 0.008 versus control group (two-way ANOVA).

Discussion

This study shows that 1) similar to ACh, VEGF induces endothelium-dependent relaxation in both SHR and WKY rat aorta; 2) the endothelium-dependent relaxation was mainly due to NO; 3) this endothelium-dependent relaxation is impaired in young SHR with developed hypertension, but not in the young SHR without hypertension, and the mechanism of this impairment may be due to decreased release of NO from the dysfunctional endothelium; 4) indomethacin enhanced VEGF- or ACh-induced relaxations, and the enhancement was greater in the adult SHR but not in the young SHR, suggesting that an increase in release of endothelium-derived contracting factors (EDCFs) through cyclo-
The endothelium is thought to produce and release various vasoactive substances, including prostacyclin, NO, endothelium-derived hyperpolarizing factor (EDHF), and EDCFs. NO is the primary factor on large conductance arteries, whereas EDHF is considered to be a major determinant of vascular caliber in small arteries and regulates the vascular resistance (Garland et al., 1995; Ge and He, 1999, 2000). Under normal conditions, the release of EDCF occurs in certain arteries. The degree of contraction or relaxation of the vascular smooth muscle cells characterizes the general vasomotor tone, which modulates the local blood pressure level and distributes the flow according to metabolic needs. The stable balance among NO, EDHF, and EDCFs released from the endothelium is disturbed by diseases such as hypertension, atherosclerosis, and diabetes. In the SHR, the endothelium-dependent relaxation induced by a variety of vasodilator agents, such as ACh, is markedly impaired. This impairment is considered to be due to a decreased release of NO, a decreased release of EDHF, or an increased release of EDCF in various arteries (Diederich et al., 1990; Fujii et al., 1992; Hayakawa et al., 1993). In the present study, the endothelium-dependent relaxation in response to both VEGF and ACh was significantly reduced in adult SHR with hypertension compared with WKY rats. This is consistent with a recent report by Brovkovych et al. (1999), who demonstrated that in the hypertensive rats the dysfunctional endothelium released 40% less NO than that of the normotensive rats. The mechanism for the decreased bioavailability of NO may be due to the higher production of \( \cdot O_2^- \) from oxygen in the SHR (Brovkovych et al., 1999).

In the present study, the relaxation induced by both ACh and VEGF was substantially enhanced by pretreatment with indomethacin in all SHR and WKY rats. Although the enhancement of this relaxation by indomethacin existed in all tested rats, including adult and young SHR and WKY rats, only in the adult SHR such enhancement was significantly greater than that in the age-matched WKY rats. These data suggest that an increase in release of EDCF may also be involved in the impairment of the endothelium-dependent relaxation in the adult SHR. Our finding is essentially in agreement with the observation by Lüscher and Vanhoucke (1986) that the reduction of ACh-induced relaxation in the adult SHR aorta might be also due to the release of EDCFs in vascular endothelium of hypertensive animals. In fact, it has been shown that the cyclooxygenase inhibitor indomethacin prevents the synthesis/release of cyclooxygenase-derived EDCF in the endothelium of rat aorta (Rubanyii et al., 1993).

A recent study has demonstrated that an EDHF-like relaxing factor may be involved in ACh-induced relaxation in SHR renal arteries (Kagota et al., 1999). In the present study, VEGF-induced endothelium-dependent relaxation was significantly reduced in the presence of indomethacin and L-NNA and the residual relaxation was abolished by the addition of NO scavenger oxyhemoglobin. This demonstrates that L-NNA could not totally inhibit the synthesis and release of NO from the endothelium of the rat aorta. By direct measurement of NO, we have recently demonstrated in the porcine coronary artery that the production of NO is not completely inhibited by even high dose of the NO synthase inhibitor L-NNA (300 \( \mu M \)) (Ge et al., 2000). In this study, we did not have direct evidence of EDHF involvement since the residual relaxation in the presence of L-NNA + indomethacin was abolished by further addition of oxyhemoglobin. This suggests that the VEGF-induced relaxation in the SHR and WKY rat aorta is NO-dependent. Furthermore, although nitroglycerin elicited nearly complete relaxation of all the aortic ring segments of both SHR and WKY rats even at the presence of L-NNA and indomethacin, oxyhemoglobin markedly attenuated this endothelium-independent relaxation (Fig. 4). Therefore, this study shows that oxyhemoglobin can scavenge the endothelium-derived NO and to some extent, NO from NO donors as well. This finding agrees with a study by Zhou and Torphy (1991) demonstrating that hemoglobin inhibited nitroglycerin-induced relaxation and cGMP accumulation in the canine trachealis.

Animal experiments and clinical studies have shown that chronic hypertension is associated with endothelial dysfunc-
tension characterized by decreased endothelium-dependent relaxations and increased endothelium-dependent contractions (Rubanyii et al., 1993; Vanhoutte and Boulanger, 1995; Rizzi et al., 1998). However, it is largely unknown whether endothelial dysfunction is the consequence or an important pathogenetic cause of hypertension. Recent studies suggest that endothelial dysfunction observed in hypertensive blood vessels might be a consequence rather than a cause of the disease process (Tesfainaram and Ogletree, 1995; Vanhoutte and Boulanger, 1995; Rizzi et al., 1998; Shimokawa, 1998). The present study demonstrates that only the adult SHR with already developed hypertension, not the young SHR at the prehypertensive stage, exhibited the impairment of endothelial-dependent relaxation. This supports the notion that endothelial dysfunction is a likely consequence of hypertension.

In conclusion, the present study demonstrates that VEGF induces endothelium- or NO-dependent relaxation, which is blunted in the adult SHR with developed hypertension. The mechanism of this impairment may be related to decreased release of NO although increased release of contracting factors from the dysfunctional endothelium may also be involved.

Acknowledgments

The technical assistance of staff in the Department of Comparative Medicine, Oregon Health Sciences University is gratefully acknowledged.

References


Browkowych V, Dobruczki LW, Browkowych S, Dobrocki I, Do Nascimento CA, Bu-...