Endothelium-Derived Contracting Factors in Resistance Arteries of Young Spontaneously Hypertensive Rats Before Development of Overt Hypertension

Michael Jameson, Fu-Xiang Dai, Thomas Lüscher, Joseph Skopec, Alice Diederich, and Dennis Diederich

Vascular relaxations are impaired in adult spontaneously hypertensive rats (SHRs) because of increased production of an endothelium-derived, cyclooxygenase-dependent contractile factor or factors. To test the hypothesis that alterations in endothelial function precede and contribute to the development of overt hypertension in SHRs, we compared in myographs endothelium-mediated relaxations of mesenteric resistance arteries from 4-week-old SHRs and Wistar-Kyoto (WKY) rats. Acetylcholine (10⁻⁸ to 10⁻⁴ M) induced comparable relaxations in SHR and WKY arteries precontracted (ED₅₀) with norepinephrine. In arteries obtained from SHRs but not from WKY rats, relaxations were replaced by contractile responses with higher concentrations of acetylcholine (10⁻⁶ to 10⁻⁵ M). The contractile responses were endothelium dependent, were augmented by nitro L-arginine (10⁻⁴ M), and were prevented by pretreatment with indomethacin (10⁻⁵ M) or 3-amino-1,2,4-triazole (10⁻³ M), an inhibitor of superoxide anion production via the cyclooxygenase pathway. Inhibition of thromboxane synthetase (CGS-13080, 5x10⁻⁵ M) and antagonism of prostaglandin H₂/thromboxane A₂ receptors (SQ-29,548, 5x10⁻⁵ M) failed to block the contractile response to acetylcholine in SHR arteries. Acetylcholine-mediated relaxations were significantly impaired in mesenteric arteries from 16-week-old SHRs but not from WKY rats. Endothelium-independent relaxations produced by sodium nitroprusside and contractile responses to norepinephrine and endothelin were comparable in arteries from SHRs and WKY rats of all ages. In summary, endothelium-dependent relaxations of mesenteric arteries from "prehypertensive" SHR rats were impaired by the production of a contractile factor (or factors) that appears to be superoxide anions.

Key Words • acetylcholine • endothelium-derived relaxing factor • endothelins • free radicals • indomethacin • vascular resistance

An increase in peripheral resistance is a characteristic finding in established essential hypertension in humans and in genetic strains of spontaneously hypertensive rats (SHRs). Peripheral resistance may increase as a result of either enhanced contractility or impaired relaxation of vascular smooth muscle of resistance arteries. Endothelial cells modulate underlying vascular smooth muscle tone by releasing endothelium-derived relaxing factor and endothelium-derived contracting factor (EDCF). An imbalanced production of relaxing and contracting factors may initiate as well as sustain the abnormal vasoconstriction of hypertension.

Endothelium-dependent relaxations of the aorta and mesenteric resistance arteries of adult SHRs are impaired. The impaired relaxations of the SHR vessels are due to the production of cyclooxygenase and EDCF, most likely prostaglandin H₂, which opposes the relaxing properties of endothelium-derived nitric oxide.

Inhibition of cyclooxygenase prevents the production of the contractile factor. Although production of EDCF was observed in arteries obtained from only SHRs in earlier studies, recent reports also describe production of an EDCF in the aorta obtained from aged Wistar-Kyoto (WKY) rats. The studies reported here were carried out to determine whether the alterations in vasoactive function of the SHR endothelium represent a genetic alteration in endothelial cell metabolism that precedes the accelerated phase of hypertension noted in this strain or represent a response of the endothelium to sustained hypertension and aging.
Methods

Experimental Animals

Male SHRs and WKY rats of 4, 16, and 28 weeks of age were obtained from Harlan Laboratories, Boston. All rats were maintained four per cage at a constant temperature (24 ± 1°C), with a 12-hour dark/light cycle and standard rat chow. All procedures followed were in accordance with institutional guidelines. Systolic blood pressures were measured in conscious, prewarmed, restrained rats by the tail-cuff method using plethysmography and a physiograph recorder (model 11 TC, Innovators in Instrumentation, Woodland Hills, Calif.). The median of four to five successive measurements was used as the estimate of blood pressure. Blood pressures and acetylcholine responses reported for 4-10-week-old SHRs were derived from a single group of SHRs obtained at age 4 weeks; blood pressures for the actual rats used in the vascular studies are presented. Rats were anesthetized with ether. The mesentery was removed en bloc through an abdominal incision and was placed in cold Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 22, edetate calcium disodium 0.026, and glucose 11. Small mesenteric arteries (first, second, and third branches as a unit) were dissected free under a dissecting microscope with care taken to avoid stretching and trauma to the vessel. A 20-μm-diameter wire was inserted into the lumen of the vessel, and a 2-mm distal segment (second branch in 4-week-old and third branch in older rats) of the vessel was transected with the wire in place. For removal of endothelium, the remaining vessel was cannulated and perfused with 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) for 45 seconds followed by Krebs bicarbonate solution; a 2-mm-long segment of the distal end was prepared as above.

Experimental Setup

The vessel segments were positioned in myographs (Living Systems, Burlington, Vt.) filled with Krebs-Ringer solution and were maintained at 37°C and pH 7.40-7.45 by aeration with 5% CO₂-95% O₂. The vessels were allowed to equilibrate for 30 minutes, after which force was applied to the vessel wall by displacement of the two mounting wires in the vessel lumen. Optimum wall tension was determined by the contraction of the two mounting wires in the vessel lumen. Vessel diameter was calculated from measurements of the internal circumference (L) was calculated from L=(Tr+2)d+2f, where f is mean distance between the inner edges of the wires. Vessel diameter was calculated as L/π. In practice, a force of 30-35 and 50-60 mg was applied to the wall of vessels obtained from 4- and 16-28-week-old rats, respectively. After an additional 30-minute equilibration, isometric response curves were generated by the addition of contracting or relaxing agonists to the bath. Each subsequent concentration-response study was carried out after a 45-60-minute period of reequilibration with no agonist. Acetylcholine-mediated responses (with and without inhibitors) were derived from the initial exposure of the study vessel to the muscarinic agonist.

Drugs

Acetylcholine hydrochloride, sodium nitroprusside, L-norepinephrine, histamine, ketoconazole, indomethacin, bovine superoxide dismutase (3,200 units/mg protein), sodium salicylate, deferoxamine, arachidonic acid, Triton X-305, bovine serum albumin, CHAPS, 3-amino-1,2,4-triazole (AT), N⁶-nitro L-arginine (L-NA), and L-arginine HCl were obtained from Sigma Chemical Co., St. Louis, Mo. 1,3-Dimethyl-2-thiourea was purchased from Aldrich Chemical Co., Milwaukee, Wis. CGS-13080 (thromboxane synthetase inhibitor) was a gift from CIBA-GEIGY, Summit, N.J. SQ-29,548, a prostaglandin H₂/thromboxane A₂ receptor antagonist, was a gift from Squibb, Princeton, N.J. Endothelin-1, obtained from Peptides International, Inc., Lexington, Ky., was dissolved in H₂O and diluted for use in 0.01% Triton X-305 containing 0.2 mg/ml bovine serum albumin. Indomethacin was dissolved in equimolar Na₂CO₃. All other drugs were dissolved in H₂O. Drug concentrations are expressed as final molar (M) bath concentrations.

Calculations and Statistics

Concentration–response curves for acetylcholine and sodium nitroprusside were obtained in a cumulative fashion (10⁻³ to 10⁻⁴ M) after stabilization of a preconcentration of the vessel with norepinephrine (50% of maximal contraction, ED₅₀ dose). Individual data points, ED₅₀ (expressed as negative logarithm of the concentration of agonist required to produce 50% relaxation, pD₂), the area under the concentration–response curve, and the maximal relaxation were used for comparative analyses. In rings in which ED₅₀ could not be calculated, a value of >4 was used. Results are presented as mean±SEM; n refers to the number of rats from which vessels were studied. Statistical evaluation was performed using Student's t test for paired or unpaired observations or one-way analysis of variance. Means were considered significantly different when the two-tailed probability was <0.05.

Results

Blood Pressure, Weights, and Vessel Dimensions

Table 1 summarizes mean±SEM values for blood pressures and body weights. Blood pressures were measured at 4, 6, and 10 weeks of age in a single group of SHRs obtained at 4 weeks of age to more clearly define the increase of blood pressure as a function of age. Mean values for blood pressures were higher in SHRs than in WKY rats at all ages. However, the small increases (6 mm Hg differences) in mean blood pressures in SHRs noted at 4 and 6 weeks of age were not significantly different from those of WKY rats. Blood pressures of 10-week-old and older SHRs were significantly higher than those of WKY rats (p<0.05). The increase in blood pressures from age 6 to 10 weeks averaged 11.5 and 2 mm Hg per week for SHRs and WKY rats, respectively (p<0.05). Mean±SEM values for lumen diameters of the vessels from SHRs and WKY rats, respectively (p<0.05). Mean±SEM values for blood pressures and body weights. Blood pressures were measured at 4, 6, and 10 weeks of age in a single group of SHRs obtained at 4 weeks of age to more clearly define the increase of blood pressure as a function of age. Mean values for blood pressures were higher in SHRs than in WKY rats at all ages. However, the small increases (6 mm Hg differences) in mean blood pressures in SHRs noted at 4 and 6 weeks of age were not significantly different from those of WKY rats. Blood pressures of 10-week-old and older SHRs were significantly higher than those of WKY rats (p<0.05). The increase in blood pressures from age 6 to 10 weeks averaged 11.5 and 2 mm Hg per week for SHRs and WKY rats, respectively (p<0.05). Mean±SEM values for lumen diameters of the vessels from SHRs and
Table 1. Blood Pressure as a Function of Age in Spontaneously Hypertensive and Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (weeks)</th>
<th>n</th>
<th>Weight (g)</th>
<th>Systolic BP (mm Hg)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>4</td>
<td>10</td>
<td>52±6</td>
<td>114±3</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>WKY</td>
<td>6</td>
<td>6</td>
<td>48±5</td>
<td>120±3</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>6</td>
<td>10</td>
<td>120±4</td>
<td>120±6</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>6</td>
<td>10</td>
<td>108±5</td>
<td>126±5</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>WKY</td>
<td>10</td>
<td>6</td>
<td>205±8</td>
<td>128±7</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>10</td>
<td>10</td>
<td>185±7</td>
<td>174±7</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>WKY</td>
<td>16</td>
<td>6</td>
<td>360±16</td>
<td>142±5</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>16</td>
<td>8</td>
<td>340±20</td>
<td>195±7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WKY</td>
<td>28</td>
<td>8</td>
<td>392±8</td>
<td>148±7</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>28</td>
<td>8</td>
<td>383±4</td>
<td>214±9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

BP, blood pressure; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats. Values are mean±SEM; p value compares systolic BP of SHRs with WKY rats at each age group using Student's t test.

WKY rats were 128±4 versus 133±10 μm and 152±5 versus 148±6 μm at 4 and 16 weeks of age, respectively.

Endothelium-Dependent Responses

Acetylcholine induced prompt relaxations of mesenteric arteries obtained from 4-week-old WKY rats and SHRs (maximum relaxation, 88±3% and 78±3%; pD2, 7.5±0.1 and 6.7±0.4; n=14 and 10, respectively) (Figure 1). However, as the acetylcholine concentration was increased above 10⁻⁷ M, the relaxations were replaced by powerful contractions in SHR arteries. Contractions in response to acetylcholine were not observed in vessels obtained from WKY rats of any age. In WKY control arteries, arachidonic acid (10⁻⁹, 10⁻⁷, and 10⁻⁵ M) produced small contractions in norepinephrine-activated arteries with endothelium (10±6%, 11±6%, and 28±11%, respectively; n=5) as well as without endothelium (6±2%, 13±10%, and 34±12%, respectively; n=5). The magnitude of the contractile response to acetylcholine in SHR arteries was not affected by the addition of arachidonic acid (10⁻⁷ M) to the bathing medium (n=3, data not shown). Prior removal of the endothelium abolished acetylcholine-induced relaxations of mesenteric resistance arteries from both WKY rats and SHRs as well as the contractile response noted in SHR vessels (n=5, data not shown).

Histamine (10⁻⁵ to 10⁻⁴ M) also produced a contractile response in SHR arteries but not in WKY arteries (Figure 2). As with acetylcholine, the responses to histamine were endothelium dependent; prior removal of the endothelium blocked histamine-induced relaxations in both SHR and WKY arteries and the contractile response in SHR arteries.

Mediators of Endothelium-Dependent Relaxations

Relaxations induced by acetylcholine were not dependent on the production of prostaglandins. Preincubation of SHR vessels with indomethacin (10⁻⁵ M) increased relaxations by blocking the contractile response induced by acetylcholine (Figures 1 and 3). After inhibition of cyclooxygenase, acetylcholine-induced relaxations were essentially identical in SHR and WKY mesenteric resistance arteries (pD2 values, 7.6±0.2 versus 7.5±0.1, respectively; p>0.1). Addition...
of hemoglobin (10^{-5} M) to the bathing solution reversed relaxations induced by acetylcholine (10^{-3} M) in both WKY and SHR vessels; maximum relaxations decreased from 88% and 80% to 10% and 12% for WKY and SHR vessels, respectively (n=5). Precontraction of the SHR arteries with norepinephrine was required for the development of endothelium-dependent contractions to acetylcholine. In quiescent SHR vessels, the maximum contractions induced by acetylcholine (10^{-9} to 10^{-4} M) averaged <5% of the maximum response to norepinephrine (n=5).

Pretreatment of SHR mesenteric arteries with L-NA (10^{-4} M) for 30 minutes before addition of acetylcholine abolished relaxations and markedly potentiated the contractile response to the muscarinic agonist (Figure 3). Indomethacin added along with L-NA abolished the contractile response to acetylcholine. The combination of L-NA and indomethacin (Indo) blocked both relaxation and the contractile response in SHR arteries. *Significantly different from control SHR arteries.

**Mediators of Endothelium-Dependent Contractions**

Additional experiments were performed to determine the mediator or mediators of the cytochrome P-450-dependent monooxygenase pathway,17 also failed to block the contractile response to acetylcholine (Figure 3).

**Potential Role of Superoxide and Hydroxyl Radicals**

Potential role of superoxide or hydroxyl radicals or both as mediators of the contractile response was tested using AT (10^{-3} M), the inhibitor of superoxide production via cyclooxygenase,18-20 and the cell-permeable hydroxyl radical scavenger21 dimethylthiourea (10^{-4} M). Preincubation of arteries from 4-week-old SHRs with AT abolished the contractile response to acetylcholine (Figure 5), whereas dimethylthiourea pretreatment had essentially no effect on the contractile response to the muscarinic agonist. The pD2 values for control, AT-, and dimethylthiourea-treated SHR vessels (6.7±0.4, 6.1±0.4, and 7.0±0.6, respectively) were not significantly different (p>0.2 for both AT and dimethylthiourea versus control). In additional experiments, deferoxamine (10^{-3} M) or sodium salicylate (10^{-4} M), scavengers of hydroxyl radicals, also failed to block the EDCF released by acetylcholine in SHR arteries (n=3, data not shown). Similarly, pretreatment of SHR arteries with superoxide dismutase (100 units/ml), a cell-impermeable scavenger of superoxide anions, also failed

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Effect of Age

Acetylcholine-mediated responses were essentially identical in arteries from 4- and 8-week-old SHRs (Figure 6). In vessels from 4-week-old SHRs, acetylcholine-induced relaxations were impaired because of an increase in the contractile response to higher concentrations of the agonist. Preincubation of arteries from 16-week-old SHRs with indomethacin (Indo, 10^{-5} M) improved relaxations and blocked the contractile response to acetylcholine. *Significantly different from 4-week-old SHR arteries.

to block the contractile response to acetylcholine (n=4, data not shown).

Endothelium-Independent Responses

Mesenteric arteries (with endothelium) from 4-week-old SHRs were more sensitive to sodium nitroprusside than arteries from age-matched WKY rats (Figure 7; pD2 values, 6.4±0.2 and 5.6±0.2, respectively; ρ<0.05). Removal of the endothelium markedly enhanced the sensitivity of arteries from both groups of rats to sodium nitroprusside; pD2 values increased to 8.1±0.1 and 8.2±0.2 for SHR and WKY arteries, respectively. Relaxations induced by sodium nitroprusside in arteries from 28-week-old SHRs and WKY rats were not significantly different from those noted in 4-week-old vessels (data not shown). Preincubation of arteries from 4-week-old SHRs with AT (10^{-3} M) or with dimethylthiourea (10^{-4} M) did not alter relaxations induced by nitroprusside; pD2 values were 6.4±0.2 (n=5) and 6.4±0.4 (n=5), respectively.

Relaxations induced by the calcium antagonist diltiazem (10^{-8} to 10^{-4} M) were virtually identical in arteries from 4-week-old SHRs and WKY rats; pD2 values were 6.1±0.2 and 5.9±0.3, respectively.

Vascular Smooth Muscle Contractility

Contractions induced by norepinephrine (10^{-7} to 10^{-4} M) and by endothelin (10^{-10} to 10^{-7} M) were not significantly different between arteries from 4-week-old SHRs and WKY rats. The pD2 values for norepinephrine and endothelin were 5.4±0.1 and 7.7±0.2 for arteries from SHRs (n=10) and 5.3±0.1 and 7.7±0.1 for WKY arteries (n=10), respectively.

Discussion

The present study demonstrates that acetylcholine induces endothelium-dependent contractions in isolated mesenteric resistance arteries of 4-week-old SHRs, whereas no such response was observed in WKY rats. Endothelium-dependent contractions have previously been demonstrated in mesenteric resistance arteries of adult, hypertensive SHRs.8,11-14 Contractions to acetylcholine were not observed in perfused arteriograph system SHR mesenteric arteries,8,10 suggesting that flow may downregulate the cyclooxygenase pathway. Endothelial dysfunction, characterized by production of one or more EDCF s in response to both acetylcholine and histamine, preceded an accelerated phase of blood pressure increase between weeks 6 and 10 in the SHR. Other investigators have described a “developmental phase” of hypertension noted between weeks 5 and 13 in SHRs.22-24 In the present study, mean systolic pressures were 6 mm Hg higher in SHRs than in WKY rats at 4 weeks of age (120±3 versus 114±3 mm Hg, respectively; ρ=0.06); at 10 weeks of age, systolic pressures were 46 mm Hg higher in SHRs (ρ<0.005). Are 4-week-old SHRs in fact “prehypertensive”? There are conflicting reports in published literature (for review, see Reference 25). In one of the most definitive studies addressing this issue, Lats and col-

**Figure 6.** Line graph depicts acetylcholine concentration-response curves for mesenteric arteries from 4-, 8-, and 16-week-old spontaneously hypertensive rats (SHR). Responses in arteries from 4- and 8-week-old rats were essentially identical; relaxations were impaired in arteries from 16-week-old rats. Preincubation of arteries from 16-week-old SHRs with indomethacin (Indo, 10^{-5} M) improved relaxations and blocked the contractile response to acetylcholine. *Significantly different from 4-week-old SHR arteries.

**Figure 7.** Line graph shows relaxation responses of mesenteric arteries from 4-week-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats to nitroprusside. SHR arteries with endothelium (endo) were significantly more sensitive to nitroprusside than WKY arteries. Removal of the endothelium markedly increased sensitivity of both SHR and WKY arteries to nitroprusside (concentration shifts, 55- and 395-fold, respectively; ρ<0.05).
leagues\textsuperscript{36} compared direct intra-arterial measurements of systolic and diastolic pressures in SHRs and WKY rats at 2–4 weeks of age. Indirect measurements of systolic pressure (tail-cuff method) were also compared with direct measurements in 4-week-old rats. By direct measurements, systolic and diastolic pressures did not differ in SHRs and WKY rats at 2 or 3 weeks of age; both values were significantly elevated in SHRs at 4 weeks of age. Values obtained from indirect measurements for systolic pressure of 4-week-old rats corresponded closely with those obtained from direct measurements (123±12 versus 131±3 mm Hg for SHRs; 114±6 versus 119±3 for WKY rats, respectively). Of interest for the present study, systolic pressures by the indirect method were not significantly different; in addition, the actual values were quite similar to those noted in the present study. Because significant increases in systolic pressures were noted only after 6 weeks of age in SHRs in the present study, we suggest that at 4 weeks of age, the SHRs were in fact “prehypertensive.” Production of the EDCF before the development of hypertension suggests that the response is not a result of endothelial injury produced by hypertension but rather is related to a preexistent alteration or alterations of the cyclooxygenase pathway of SHR endothelial cells. Indeed, inhibition of cyclooxygenase or removal of the endothelium blocked the EDCF response to acetylcholine. The nature of the EDCF released by histamine awaits further studies.

A putative EDCF released by acetylcholine from SHR mesenteric resistance arteries reversed the endothelium-dependent relaxation induced by the muscarinic agonist. The latter response must be mediated by endothelium-derived nitric oxide, as it was prevented by L-NA\textsuperscript{27,28} and hemoglobin.\textsuperscript{29} After inhibition of cyclooxygenase, acetylcholine-mediated relaxations of SHR and WKY arteries were essentially identical. Thus, the impaired relaxations in SHR arteries were not due to a decreased production of nitric oxide but rather were due to the concomitantly released factor reversing its effects.

Endothelium-dependent contractions produced by acetylcholine have been demonstrated in canine basilar arteries,\textsuperscript{30} in the pulmonary artery of rabbits,\textsuperscript{31} in canine and human veins,\textsuperscript{32–35} in the aorta and renal artery of SHRs,\textsuperscript{11,36} and more recently in the aorta of very old WKY rats.\textsuperscript{13,14} Under all conditions, the contractile responses induced by acetylcholine were blocked by cyclooxygenase inhibition, a response shared by the SHR mesenteric resistance artery described in this report.

The impairment of endothelium-dependent relaxation of SHR mesenteric arteries was more marked in older SHRs. The progressive impairment in endothelium-mediated relaxation with age may well be related to secondary effects of hypertension on endothelial function.\textsuperscript{5,6,10,36} Indeed, endothelium-dependent relaxation of conduit arteries is impaired in genetic models of hypertension (SHRs and Dahl rats) as well as in models of acquired hypertension, including coarctation of the aorta, constriction of the renal artery, and mineralocorticoids plus salt.\textsuperscript{37–41} Correction of the hypertension by removing the renal produce or aortic constriction or by removing sodium from the diet restores endothelium-dependent relaxation under these conditions.\textsuperscript{37,38}

The nature of the putative cyclooxygenase-dependent constricting substance released by acetylcholine from the endothelium of SHR mesenteric resistance arteries has been partially defined using specific inhibitors of cyclooxygenase products. Products of the cyclooxygenase pathway known to produce vascular contractions include prostaglandin H\textsubscript{2}, thromboxane A\textsubscript{2}, prostaglandin F\textsubscript{2\alpha}, and in the rat, prostaglandin E\textsubscript{2}. Inhibition of thromboxane synthetase (CGS-13080) and blockade of prostaglandin H\textsubscript{2}/thromboxane A\textsubscript{2} receptors (SQ-29,548)\textsuperscript{39} had no effect on the contractile response; thus, it is unlikely that either thromboxane A\textsubscript{2} or prostaglandin H\textsubscript{2} is involved. The rapidity with which the contractile responses to acetylcholine developed or disappeared tends to exclude prostaglandin F\textsubscript{2\alpha} as the responsible factor. The inability of ketoconazole to block the contractile response suggests that products of the P-450 epoxygenase pathway, such as 5,6-epoxyeicosatrienoic acid or 20-hydroxyeicosatetraenoic acid, which may be further metabolized to endoperoxide derivatives via the cyclooxygenase pathway,\textsuperscript{42,43} are not responsible for the response. Inhibition of the contractions to acetylcholine in SHR arteries by AT, an agent that inhibits superoxide production by the cyclooxygenase pathway,\textsuperscript{16–20} suggests that superoxide anions may mediate the response. The present observations do not exclude the possibility that AT may function indirectly as a cyclooxygenase inhibitor. As pointed out below, the cyclooxygenase activity of prostaglandin endoperoxide synthase is activated by free radicals. AT may inhibit activation of cyclooxygenase indirectly by altering the production of free radicals required to activate cyclooxygenase. We have no direct evidence that superoxide radicals per se serve this function. Failure of superoxide dismutase to block the contractile response in SHR arteries may be due to the inability of this large molecule to penetrate the endothelial cells producing the superoxide radicals. Superoxide anions may alter endothelial function by several mechanisms. Superoxide accelerates the inactivation of nitric oxide,\textsuperscript{44,45} thereby opposing nitric oxide–mediated modulation of basal as well as activated vascular tone. In some vessels, superoxide serves as a vasoconstricting agent, seemingly independent of its effect on nitric oxide.\textsuperscript{46} Additionally, superoxide anions can react with nitric oxide radicals to form peroxynitrite.\textsuperscript{47–50} Peroxynitrite itself may serve as a damaging radical and also may be further metabolized to the highly reactive hydroxyl radicals.\textsuperscript{18,46,51} Endothelium-dependent responses attributed to hydroxyl radicals have been described in cerebral arteries of cats\textsuperscript{18,51,52} and in the aorta of rats.\textsuperscript{53} Thus, enhanced endothelial production of superoxide anions may impair endothelial and vascular smooth muscle function via multiple mechanisms. Based on results of studies in this report, the major effect of superoxide (EDCF) appears to be “inactivation” of nitric oxide: inhibition of superoxide production by either cyclooxygenase inhibition (indomethacin) or by AT enhanced nitric oxide–mediated relaxations. We found no support for enhanced production of hydroxyl radicals serving as the EDCF. Dimethylthiourea, an effective scavenger of hydroxyl radicals that readily enters the cellular milieu where reactive radicals are generated, as well as sodium salicylate and defereroxamine, all failed to block the contractile response or to significantly improve endothelium-de-
dependent relaxation. Similarly, the potentiation of the contractile response by L-NA, an inhibitor of nitric oxide production, suggests that peroxynitrite formation is not involved in the contractile response. The fact that acetylcholine did not evoke endothelium-dependent contractions in quiescent preparations, but reversed the relaxations to acetylcholine in precontracted vessels, would suggest that the radicals formed after activation of cyclooxygenase function by inactivation of nitric oxide rather than by production of direct contractions. Contrasting findings were noted in the aorta of the SHR, where endothelium-dependent contractions have been ascribed to the formation of prostaglandin H₂, and where acetylcholine causes pronounced endothelium-dependent contractions in quiescent preparations. Contrasting findings are also noted in renal resistance arteries of the SHR, where acetylcholine induces cyclooxygenase- and endothelium-dependent contractions in precontracted arteries that are prevented by blockade of prostaglandin H₂/thromboxane A₂ receptors with SQ-29,548. A characteristic shared by the different contractile factors noted in SHR arteries is their cyclooxygenase dependence. It is conceivable that the explanation for the different mediators of the endothelium-mediated contraction in SHR arteries lies in differences in substrates for the prostaglandin H synthase reaction or in the capacities of the arteries to form nitric oxide.

Prostaglandin H synthase (prostaglandin endoperoxide synthase) is a hemoprotein that has two inseparable activities, namely, cyclooxygenase responsible for the oxidation of arachidonic acid to the hydroperoxide prostaglandin G₂, and hydroperoxidase responsible for the peroxidation of prostaglandin G₂ to prostaglandin H₂. Free radical species formed by the peroxidase reaction (synthase tyrosyl radical), by abstracting an allylic hydrogen from the substrate polyunsaturated fatty acid, may initiate the cyclooxygenase reaction by producing a fatty acyl radical capable of reacting with molecular oxygen. Indomethacin inhibits cyclooxygenase by producing one or more perturbations of the hydroperoxide-induced radical species required to activate cyclooxygenase. Recent studies suggest that nitric oxide (or the derivative nitroxylic anion) may function as an antioxidant that prevents activation of cyclooxygenase.

Nitric oxide can form a nitric oxide–heme adduct with the iron atom of cyclooxygenase, thereby converting the ferric-active enzyme to the ferrous-active form of the enzyme. The ferrous forms of cyclooxygenase are inactive and need to be activated to the ferric form by traces of hydroperoxides or hydrogen peroxide to function. Endothelial cells with limited production of nitric oxide may be less capable of regulating cyclooxygenase activity and indirectly the resulting endoperoxide (prostaglandin H₂) and superoxide (or other radical) production. Prostaglandin H synthase can also generate superoxide radicals when acting on arachidonic acid in the presence of NADH or NADPH. Superoxide anions accelerate the breakdown of nitric oxide but may also combine with nitric oxide to produce the peroxynitrite anion (ONOO⁻) and serve as precursors for hydroxyl radicals. Membrane fatty acid metabolism is accelerated in SHR arteries; point mutations in the phospholipase-δ gene in SHRs may be causally related to the augmented phospholipase C activity noted in SHRs. Augmentation of phospholipase C activity, by increasing intracellular calcium, could favor vasoconstriction. Similarly, acetylcholine, by increasing cellular calcium, may further increase arachidonate and phospholipid metabolism in SHR endothelial cells. Products of the activated prostaglandin H synthase pathway may function directly as contractile factors (prostaglandin H₂, thromboxane A₂, superoxide radicals) or indirectly as contractile factors by inactivation of nitric oxide (superoxide anions). Acetylcholine in all likelihood does not serve as a physiological regulator of in vivo endothelial cell vasoreactive metabolism. The relevance of the present in vitro studies arises from the fact that this muscarinic receptor agonist may serve as a useful probe for the examination of functional alterations in membrane receptors in SHR endothelial cells that may at least contribute to the development of hypertension in this genetic model.

Insummary, endothelium-dependent relaxations induced by acetylcholine are impaired in mesenteric resistance arteries of prehypertensive as well as hypertensive SHRs. Acetylcholine releases an endothelium-derived factor that reverses the effects of nitric oxide in the SHR, whereas no such substance is produced in resistance arteries obtained from normotensive WKY rats. The production of this factor requires the activity of cyclooxygenase, as indomethacin normalizes endothelium-dependent relaxations in resistance arteries of the SHR. In contrast to the aorta or renal resistance arteries of SHRs, the cyclooxygenase product or products interfering with the effects of nitric oxide in SHR mesenteric arteries is not prostaglandin H₂ or thromboxane A₂ but rather are oxygen-derived free radicals that are most likely superoxide anions. Enhanced production of endothelium-derived free radicals in the SHR may contribute to the development of hypertension, especially because this defect precedes the accelerated increase in blood pressure in this genetic model of hypertension.

References

Endothelium-Derived Contracting Factor and the SHR


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