Vascular Reactivity During the Progression of Atherosclerotic Plaque

A Study in Watanabe Heritable Hyperlipidemic Rabbits

Frank D. Kolodgie, Renu Virmani, Holly E. Rice, and Wolfgang J. Mergner

The effects of varying degrees of atherosclerotic plaque on vascular responsiveness in aortas of Watanabe heritable hyperlipidemic (WHHL) rabbits and New Zealand White (normal cholesterolemic) rabbits were studied. Ring segments from the aortic arch and thoracic aorta were mounted in organ chambers for isometric tension recording and measurement of endothelium-derived relaxing factor. WHHL rabbits were divided into three groups according to age: group 1, 3–5 months; group 2, 6–9 months; and group 3, 12–14 months. Atherosclerotic changes (expressed as a percent of total surface area) in the aortic arches in groups 1, 2, and 3 were 11±3% (mild), 28±6% (moderate), and 54±8% (severe) respectively; only occasional plaques were present in the thoracic aorta in all groups. Maximal contractions elicited with phenylephrine progressively decreased with increasing degrees of atherosclerotic plaque. Contractions evoked by histamine were augmented in all groups of WHHL rabbits when compared with controls, whereas those to serotonin were augmented only in vessels with mild atherosclerosis. As the severity of the intimal lesions increased, endothelium-dependent relaxations to acetylcholine, ATP, and calcium ionophore A23187 progressively decreased. Endothelium-independent relaxation to nitroglycerin was virtually complete in all segments. However, vessels with severe atherosclerosis were less sensitive to this agent as illustrated by a significant increase in the ED50 value. Scanning electron microscopy revealed a predominant loss of endothelial cells in the central regions of fibrous plaques. Thus, in WHHL rabbits, hypercholesterolemia and atherosclerosis result in an increased responsiveness of vascular smooth muscle to histamine and serotonin. Endothelium-mediated relaxation of vascular smooth muscle is reduced with the progression of atherosclerosis primarily due to a loss of endothelial cells. (Circulation Research 1990;66:1112–1126)

Coronary spasm plays an essential role in the pathophysiology of ischemic heart disease. In patients with variant angina, coronary spasm has been observed angiographically after administration of certain pharmacological agents. Coronary vasospasm usually occurs at the sites of varying degrees of atherosclerosis. Although the clinical presentation is well defined, the mechanism underlying vasospasm is not well understood. Recently, it has become evident that endothelial cells selectively modulate vascular smooth muscle tone by releasing a potent substance, endothelium-derived relaxing factor. Endothelial cell function may become impaired in atherosclerotic vessels, and the absence of vasodilatory responses may render the vessel defenseless against certain vasoconstrictor stimuli. Numerous animal models of diet-induced hypercholesterolemia with and without balloon injury have demonstrated attenuation of endothelium-mediated relaxation and also have augmented constrictor responses to various neurohumoral agents both in vivo and in vitro. Similar findings have been reported in isolated human coronary arteries.

The purpose of this investigation was to evaluate the effects of the progression of atherosclerosis on vascular reactivity in the aortas of Watanabe heritable hyperlipidemic (WHHL) rabbits and to determine if impairment or loss of endothelial cells is the mechanism underlying altered vascular responsiveness. This model was chosen because it exhibits vascular lesions morphologically comparable with human atherosclerosis.
Materials and Methods

Animal Model of Atherosclerosis

Homozygous WHHL rabbits were obtained from our breeding colony at the Armed Forces Institute of Pathology, Washington, DC. These animals exhibit consistent hereditary hyperlipidemia as a result of inbreeding.\textsuperscript{21} WHHL rabbits of either sex, aged 3–5 months (group 1, n=5), 6–9 months (group 2, n=6), and 12–14 months (group 3, n=5) and weighing approximately 2–3.2 kg, were studied. New Zealand White rabbits, aged 5–6 months (n=5) and 10–14 months (n=4), were studied as controls. In the homozygous WHHL rabbit model, the aortic arch is more susceptible to atherosclerotic plaque formation than the thoracic aorta. All animals were fed standard rabbit chow (Purina, St. Louis, Missouri). The care of rabbits used in the study complied with the guidelines of the National Institutes of Health on animal experimentation.

Measurement of Serum Cholesterol and Triglycerides

Serum cholesterol and triglycerides were measured enzymatically with an automated method.\textsuperscript{22,23}

Vascular Reactivity Studies

Before study, rabbits were fasted for 12 hours. Animals were anesthetized with sodium pentobarbital (25–30 mg/kg i.v.). A blood sample was obtained for determination of serum cholesterol and triglyceride levels. Rabbits were then killed by exsanguination over a 5–10-minute period. The aortic arch and thoracic aorta were carefully removed and immediately placed in modified Krebs-Henseleit buffer (KHB) containing (mM) NaCl 118.3, KCl 4.7, CaCl\textsubscript{2} 2.5, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25.0, calcium EDTA 0.026, and glucose 11.1 at 37° C and bubbled with a mixture of 95% O\textsubscript{2}/5% CO\textsubscript{2}.

Vessels were cleaned of adherent fat and connective tissue, cut into 3–5-mm ring segments, and suspended vertically with suture in an organ bath with 30 ml oxygenated KHB maintained at 37° C. The interval between the removal of the aorta from the animal to the suspension of the aortic rings in organ baths did not exceed 20 minutes. One additional ring from the aortic arch and thoracic aorta of each rabbit was immediately fixed in McDowell-Trump (formaldehyde/glutaraldehyde) solution (for details, see below) for later assessment of intimal surfaces by scanning electron microscopy to determine if the experimental time course affected vessel morphology.

All studies were performed in the presence of propranolol (0.1 \mu M). Tension was monitored continuously with a linear force transducer (model B-25, Gould, Cleveland, Ohio). Aortic segments were passively stretched to a resting tension of 2 g over a 90-minute period. In some segments of the arch and thoracic aorta, the endothelium was removed by gently rubbing the intimal surface with a cotton swab.

Four WHHL rabbits, aged 10–14 months, were perfusion-fixed with McDowell-Trump fixative. The method for perfusion fixation was similar to that described by Rosenfeld et al,\textsuperscript{24} with minor modifications. The carotid artery was cannulated and perfused with lactated Ringer's solution at 100 mm Hg pressure. The inferior vena cava was exposed by midline abdominal incision, and a surgical incision was made in the anterior wall of the vena cava. This allowed a flow rate of approximately 60–80 ml/min. Lactated Ringer's solution was infused until the runoff was free of blood. This was followed by perfusion-fixation with McDowell-Trump solution for 20 minutes. The aorta was then carefully removed, and 3–5-mm rings were cut from the ascending and thoracic aorta for scanning electron microscopy.

Protocols

Responses to vasoactive stimuli were examined in six rings from each animal (aortic arch, n=3; thoracic aorta, n=3). One ring from the aortic arch and one from the thoracic aorta of each animal underwent mechanical endothelial denudation.

Vasoconstrictor responses to phenylephrine (10\textsuperscript{-9}–10\textsuperscript{-5} M), histamine (10\textsuperscript{-5}–10\textsuperscript{-4} M), and serotonin (10\textsuperscript{-6}–10\textsuperscript{-5} M) were determined in all groups. After precontraction with a concentration of phenylephrine equivalent to the ED\textsubscript{50} of this agent, endothelium-dependent relaxation responses to ATP (10\textsuperscript{-6}–10\textsuperscript{-5} M), acetylcholine (10\textsuperscript{-9}–10\textsuperscript{-5} M), and calcium ionophore A23187 (10\textsuperscript{-6}–10\textsuperscript{-3} M) and endothelium-independent relaxation responses to nitroglycerin (10\textsuperscript{-2}–10\textsuperscript{-5} M) were studied. Dose-response curves to the various drugs were randomized. This helped minimize the possibility that time in the organ bath was a factor when the tissue was exposed to an agent or that previous exposure to a drug interfered with the subsequent pharmacological response of the agonist tested. After each concentration-response curve, vessels were washed repeatedly with KHB and allowed to equilibrate for a minimum of 30 minutes.

Gross Morphological Examination

At the completion of each experiment, the rings were washed with KHB and allowed to equilibrate to baseline resting tension. Vascular segments were then removed from the apparatus, cut open longitudinally, and pinned stretched on a tongue depressor with the endothelial surface exposed. Specimens were fixed overnight in McDowell-Trump solution at 4° C. The specimens were then photographed under fixative. Photographic slides were projected (×10), and the total surface and atherosclerotic plaque areas were quantitated by using computerized planimetry.

Histological Examination

A 1–2-mm longitudinal section was cut from each ring segment for light microscopy. The remaining tissue was submitted for scanning electron microscopy. A few samples were selected for transmission electron microscopy.
Light Microscopy

Tissue samples were dehydrated and embedded in paraffin on edge (cross section), cut into 4-μm sections, and stained with hematoxylin-eosin and Movat pentachrome stain. Histological sections were projected (×25), and the intima-media index (intima/media area times 100) was determined by computerized planimetry.

Scanning Electron Microscopy

Tissue samples were dehydrated and critical-point-dried with liquid carbon dioxide, mounted on stubs, coated with gold palladium, and visualized on a scanning electron microscope (model S-570, Hitachi Instruments, Danbury, Connecticut). Each vascular segment was examined for the presence or absence of endothelial cells, mononuclear adherence, and the extent of atherosclerosis. The percent surface area covered by endothelium was estimated visually at ×150 magnification. Areas where the vessel was in contact with the suture were not evaluated for endothelial cell loss.

Drug Preparation

The pharmacological agents used were acetylcholine chloride (Sigma Chemical, St. Louis, Missouri), ATP (Sigma Chemical), calcium ionophore A23187 (Sigma Chemical), histamine (Sigma Chemical), nitroglycerin (Marion Laboratories, Kansas City, Missouri), phenylephrine (Sigma Chemical), propranolol HCL (Sigma Chemical), and 5-hydroxytryptamine creatinine sulfate (serotonin, Sigma Chemical). All drugs were freshly prepared using distilled water except for A23187, which was dissolved in dimethyl sulfoxide (1%). Agents were kept on ice during the experiments.

Statistical Methods

Data are expressed as mean±SEM. Relaxation responses are expressed as the percent relaxation from the amount of preconstriction produced by phenylephrine. ED50 was defined as the concentration of agonist at which 50% of the maximal response was obtained. Statistical evaluation of the data was performed by Student’s t test for unpaired observations. A value of p<0.05 was considered significant.

Table 1. Serum Cholesterol and Triglyceride Levels

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>Group 1 (n=5)</th>
<th>Group 2 (n=6)</th>
<th>Group 3 (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>30.3±0.8</td>
<td>722.4±34.6</td>
<td>750.0±92.3</td>
<td>494.6±135.4</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>51.7±2.3</td>
<td>619.4±222.0</td>
<td>456.6±65.6</td>
<td>425.4±63.7</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Control, New Zealand White rabbits; group 1, Watanabe heritable hyperlipidemic (WHHL) rabbits aged 3–5 months; group 2, WHHL rabbits aged 6–9 months; group 3, WHHL rabbits aged 12–14 months.

Results

Serum Cholesterol and Triglyceride Levels

Table 1 is a comparison of the mean distribution of total serum cholesterol and triglycerides in control and WHHL rabbits. Total serum cholesterol and triglyceride levels were markedly elevated in WHHL rabbits as compared with control rabbits. Serum cholesterol and triglyceride concentrations in WHHL rabbits were maximal in the 3–5-month-old group and decreased with the age of the animal.

Vascular Responses in Control (New Zealand White) Rabbits

Phenylephrine (10^-6–10^-5 M), histamine (10^-7–10^-4 M), and serotonin (10^-8–10^-5 M) elicited concentration-dependent contractions in control segments of the aortic arch and thoracic aorta (Figure 1, Table 2). Mechanical removal of endothelial cells did not significantly alter the maximal response or ED50 value to these agonists.

During sustained contractions evoked by phenylephrine (ED50-ED90 range), acetylcholine (10^-9–10^-6 M), ATP (10^-7–10^-4 M), calcium ionophore A23187 (10^-10–10^-6 M), and nitroglycerin (10^-9–10^-6 M) induced concentration-dependent relaxations in all control vessels (Figures 2 and 3, Table 3). After mechanical removal of the endothelial cell layer, relaxation to acetylcholine, ATP, and A23187 was virtually abolished (data not shown). In contrast, vascular relaxation in response to nitroglycerin was unaffected in vessels denuded of endothelium (data not shown). There were no statistical differences in any vascular reactivity responses between the 5–6-month-old and 10–14-month-old New Zealand White rabbits (Tables 2 and 3).

Vascular Responses in WHHL Rabbits

Increasing concentrations of phenylephrine, histamine, and serotonin in WHHL rabbits, which were identical with concentrations in control animals, induced contractions in segments of the aortic arch and thoracic aortas (Figure 1, Table 2). The maximal contractile response caused by phenylephrine was significantly decreased in vessels with moderate and severe atherosclerosis when compared with vessels of control tissues with intact endothelium (Figure 1, Table 2). Maximal contractions produced by histamine were significantly augmented in both the aortic
arch and thoracic aorta and were independent of atherosclerotic plaque progression (Figure 1, Table 2). For serotonin, the concentration-response relation in vessels with minimal atherosclerosis exhibited a marked leftward shift with a significantly increased maximum constrictor response and a lower ED$_{50}$ value when compared with control rings; however, response of vessels with moderate and severe atherosclerosis was equal to control vessels (Figure 1, Table 2).

Responses to the endothelium-dependent vasodilators acetylcholine, ATP, and A23187 were similar in vessels from groups 1 and 2 WHHL rabbits when compared with control rabbits (Figure 2, Table 3). In contrast, relaxations to these agonists in the aortic arch and thoracic aorta obtained from group 3 WHHL rabbits were markedly attenuated compared with the relaxations observed in control rabbits (Figure 2, Table 3); these differences were not illustrated by a significantly decreased ED$_{50}$ value. The magnitude of the maximal relaxations (percent of initial phenylephrine-induced contractions) elicited by acetylcholine in aortic arch vessels from WHHL rabbits correlated significantly with the degree of atherosclerotic plaque and endothelial cell loss ($r^2=0.65$ and $r^2=0.73$, respectively; Figure 3) and to a lesser degree with intimal medial index ($r^2=0.47$). The maximal relaxation with high concentrations of nitroglycerin (>10$^{-6}$ M) was not different among all vessels studied (Figure 4, Table 3). However, at lower concentrations, group 1 WHHL tissues were more sensitive to nitroglycerin than moderate to severely atherosclerotic vessels (Figure 4, Table 3).

**Morphological Evaluation of Ring Segments From Control and WHHL Animals**

No visible atherosclerotic lesions were detected in any vessels removed from New Zealand White rabbits (Table 4). Of the vessels from WHHL rabbits, the aortic arch was more severely affected by atherosclerosis than was the thoracic aorta, and plaque progression increased with the age of the animal. Fatty streaks were the predominant lesion in younger animals, whereas fibrous plaques were more extensive in older animals (Figure 5 A–C). The fatty streaks...
TABLE 2. Evaluation of Contractile Responses in Control and Watanabe Heritable Hyperlipidemic Rabbits

<table>
<thead>
<tr>
<th>Phenylephrine</th>
<th>Control 1 AA</th>
<th>Control 1 TA</th>
<th>Group 1 AA</th>
<th>Group 1 TA</th>
<th>Group 2 AA</th>
<th>Group 2 TA</th>
<th>Group 3 AA</th>
<th>Group 3 TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells present (n=20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (g)</td>
<td>4.5±0.4</td>
<td>3.9±0.3</td>
<td>4.7±0.5</td>
<td>4.50±0.3</td>
<td>3.9±0.4</td>
<td>3.3±0.3</td>
<td>3.4±0.4*</td>
<td>3.7±0.6</td>
</tr>
<tr>
<td>ED50 (×10^-7 M)</td>
<td>5.8±1.3</td>
<td>5.2±0.9</td>
<td>4.5±1.1</td>
<td>3.8±0.5</td>
<td>4.3±0.6</td>
<td>3.4±0.3</td>
<td>5.4±1.7</td>
<td>3.2±0.9</td>
</tr>
<tr>
<td>Endothelial cells absent (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (g)</td>
<td>4.0±0.2</td>
<td>4.3±0.7</td>
<td>4.4±0.6</td>
<td>4.3±0.2</td>
<td>3.0±0.4</td>
<td>3.3±0.3</td>
<td>2.3±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>ED50 (×10^-7 M)</td>
<td>3.6±1.2</td>
<td>1.9±0.3</td>
<td>2.2±0.2</td>
<td>1.5±0.1</td>
<td>2.6±0.6</td>
<td>1.5±0.2</td>
<td>2.1±0.7</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td>Histamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells present (n=20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (g)</td>
<td>3.4±0.8</td>
<td>4.3±0.8</td>
<td>5.1±0.6</td>
<td>4.7±0.6</td>
<td>6.1±0.5*</td>
<td>5.7±0.2*</td>
<td>5.4±0.2*</td>
<td>6.6±0.3*</td>
</tr>
<tr>
<td>ED50 (×10^-5 M)</td>
<td>2.6±0.4</td>
<td>2.6±0.5</td>
<td>2.0±0.7</td>
<td>1.6±0.5</td>
<td>1.3±0.2</td>
<td>1.0±0.08</td>
<td>1.6±0.2</td>
<td>0.7±0.08*</td>
</tr>
<tr>
<td>Endothelial cells absent (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (g)</td>
<td>3.6±0.8</td>
<td>4.2±1.4</td>
<td>5.4±1.1</td>
<td>4.8±0.7</td>
<td>6.0±0.4</td>
<td>6.1±0.4</td>
<td>5.2±1.0</td>
<td>5.4±0.7</td>
</tr>
<tr>
<td>ED50 (×10^-5 M)</td>
<td>2.2±0.6</td>
<td>2.3±0.8</td>
<td>2.8±1.2</td>
<td>0.9±0.4</td>
<td>0.7±0.08</td>
<td>0.9±0.12</td>
<td>0.7±0.09</td>
<td>0.4±0.01</td>
</tr>
<tr>
<td>Serotonin (5-HT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells present (n=20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (g)</td>
<td>3.0±0.5</td>
<td>2.0±0.4</td>
<td>1.6±0.4</td>
<td>1.2±0.6</td>
<td>4.8±0.3*</td>
<td>4.2±0.3*</td>
<td>2.7±0.2</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>ED50 (×10^-6 M)</td>
<td>1.4±0.2</td>
<td>1.6±0.3</td>
<td>1.1±0.4</td>
<td>1.6±0.5</td>
<td>0.3±0.02*</td>
<td>0.2±0.02*</td>
<td>1.5±0.6</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Endothelial cells absent (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (g)</td>
<td>2.7±0.6</td>
<td>3.4±0.6</td>
<td>3.3±0.3</td>
<td>2.0±0.6</td>
<td>4.8±0.4</td>
<td>4.6±0.2</td>
<td>2.4±0.6</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>ED50 (×10^-6 M)</td>
<td>1.2±0.4</td>
<td>1.4±0.5</td>
<td>1.3±0.5</td>
<td>1.8±0.8</td>
<td>0.2±0.03</td>
<td>0.4±0.09</td>
<td>1.0±0.3</td>
<td>0.9±0.3</td>
</tr>
</tbody>
</table>

Values are reported as mean±SEM. Control 1, New Zealand White rabbits aged 5–6 months; control 2, New Zealand White rabbits aged 10–14 months; group 1, Watanabe heritable hyperlipidemic (WHHL) rabbits aged 3–5 months; group 2, WHHL rabbits aged 6–9 months; group 3, WHHL rabbits aged 12–14 months; AA, aortic arch; TA, thoracic aorta; Max, maximum contractile response; n, number of ring segments in each group.

*Significantly different from control rings (endothelial cells present) at p<0.05 by Student's t test for unpaired observations.

<figure>
<figcaption>FIGURE 2. Graphs showing effect of acetylcholine, ATP, and Ca^2+ ionophore A23187 (endothelium-dependent relaxation) on unrubbed ring segments from the aortic arch and thoracic aorta of control rabbits (✓) and Watanabe heritable hyperlipidemic rabbits aged 3–5 months (☐), 6–9 months (△), and 12–14 months (○) during contractions to phenylephrine (PE) in the ED50–ED90 range of this agonist. The results are mean±SEM. Filled symbols (■,●) indicate statistically significant differences (p<0.05) compared with control rings.</figcaption>
</figure>
lesions were formed mainly by foam cells, smooth muscle cells, and connective tissue. Fibrous atherosclerotic plaques consisted of central areas of pultaceous debris with surrounding foam cells, mucopolysaccharides, smooth muscle cells, and collagen (Figure 5C). Focal areas of calcification were seen in the oldest animals with fibrous plaques. The thoracic aorta showed focal lesions (predominantly fatty plaques) around vertebral artery branch points (Figure 5D).

Scanning electron microscopy in vessels without atherosclerosis revealed intact endothelial cells that were well aligned (Figure 6). Early atherosclerotic lesions consisted of focal monocytes adhering to endothelial cells at various stages of spreading and invagination (Figure 7); older lesions showed numerous monocytes adhering and spreading over the endothelial surface (Figure 8). Plaque progression showed lobulated lesions with stretched endothelium overlying subendothelialized monocytes. These raised lesions were often disrupted with focal loss of endothelium exposing underlying monocytes (Figure 9). Fibrous plaques showed central regions of focal loss of endothelium or large deendothelialized surfaces (Figures 9 and 10). All ring segments that underwent mechanical denudation demonstrated a near total loss of endothelium as confirmed by scanning electron microscopy (Figure 11). No differences in endothelial cell preservation were noted between vessels in the bath versus those fixed immediately after the animal was killed or when aortas were perfusion-fixed in situ.

The two 10-month-old perfusion-fixed rabbits showed raised lesions with monocyte adherence and occasional endothelial retraction in areas of fatty streaks. The two 14-month-old rabbits showed raised atherosclerotic plaques with areas of focal endothelial loss with monocyte, platelet, and occasional red cell adherence. At the edges of the raised atherosclerotic plaques, monocyte adherence was extensive. Focally, the raised fatty plaques showed endothelial retraction exposing the underlying foam cells. Also, fibrous

---

**TABLE 3. Evaluation of Relaxation Responses in Control and Watanabe Heritable Hyperlipidemic Rabbits**

<table>
<thead>
<tr>
<th></th>
<th>Control 1</th>
<th>Control 2</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (%)</td>
<td>72±6</td>
<td>92±5</td>
<td>85±4</td>
<td>87±4</td>
<td>73±7</td>
</tr>
<tr>
<td>ED90 (×10⁻⁸ M)</td>
<td>4.8±1.3</td>
<td>6.2±1.8</td>
<td>3.8±1.1</td>
<td>5.7±1.8</td>
<td>10.5±1.6*</td>
</tr>
<tr>
<td>CA²⁺ ionophore</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (%)</td>
<td>75±3</td>
<td>90±3</td>
<td>89±3</td>
<td>92±3</td>
<td>74±3</td>
</tr>
<tr>
<td>ED90 (×10⁻⁸ M)</td>
<td>2.8±0.5</td>
<td>4.2±1.2</td>
<td>2.3±0.2</td>
<td>4.5±1.9</td>
<td>4.6±1.2</td>
</tr>
<tr>
<td>ATP (n=20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (%)</td>
<td>62±5</td>
<td>60±6</td>
<td>67±7</td>
<td>65±7</td>
<td>55±8</td>
</tr>
<tr>
<td>ED90 (×10⁻⁵ M)</td>
<td>0.7±0.2</td>
<td>2.1±0.5</td>
<td>1.8±0.8</td>
<td>0.8±0.2</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (%)</td>
<td>99±4</td>
<td>97±4</td>
<td>84±5</td>
<td>91±4</td>
<td>100</td>
</tr>
<tr>
<td>ED90 (×10⁻⁸ M)</td>
<td>2.6±0.7</td>
<td>2.0±0.3</td>
<td>3.6±0.9</td>
<td>3.7±0.8</td>
<td>2.1±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Control 1, New Zealand White rabbits aged 5–6 months; control 2, New Zealand White rabbits aged 10–14 months; group 1, Watanabe heritable hyperlipidemic (WHHL) rabbits aged 3–5 months; group 2, WHHL rabbits aged 6–9 months; group 3, WHHL rabbits aged 12–14 months; AA, aortic arch; TA, thoracic aorta; Max, maximum relaxation (percent of phenylephrine-induced contraction); n, number of ring segments for each group.

*Significantly different from control rings at p<0.05 by Student's t test for unpaired observations.

---

**FIGURE 3.** Graphs showing correlation between the maximum relaxation evoked by acetylcholine (Ach on figure) and the percent atherosclerotic plaque (left panel) and percent endothelial loss (right panel) in unrubbed ring segments from the aortic arch of Watanabe heritable hyperlipidemic rabbits aged 3–5 months (□), 6–9 months (△), and 12–14 months (○). The response to Ach is expressed as a percent of the initial contraction evoked by phenylephrine (PE) (ED₉₀–ED₁₀ range of this agonist).
plagues showed central regions of focal loss of endothelium. These were seen only in the ascending aorta.

**Discussion**

**Present Study**

WHHL rabbits were grouped according to age and compared with New Zealand White rabbits (controls). Mean plaque area in the aortic arch and thoracic aorta increased with age progression. Maximal contractile responses to phenylephrine were decreased in vessels obtained from WHHL rabbits (groups 2 and 3) compared with control rings. Vasoconstrictor effects of serotonin were augmented in WHHL rabbits aged 3–5 months in both the aortic arch and thoracic aorta as compared with control rings, whereas responses in vessels from older WHHL rabbits with moderate and severe atherosclerosis (groups 2 and 3, respectively) were similar to controls. In contrast, constrictor responses to histamine were augmented in all groups of WHHL rabbits when compared with controls.

In the aortic arch from WHHL rabbits with moderate and severe atherosclerosis, there was a gradual attenuation of endothelium-dependent relaxation to the endothelium-dependent vasodilators. Endothelial surfaces outside the regions of fatty streaks were normal in appearance. Endothelial disruption in the aortic arch of groups 1 and 2 was primarily focal (2±1% and 12±4%, respectively), whereas in group 3, there was significant loss of endothelial cells in the central regions of fibrous plaques (36±4%). The magnitude of maximal relaxation, by acetylcholine, in aortic arch vessels from WHHL rabbits correlated significantly with the percent of atherosclerotic plaque (r²=0.65) and percent loss of endothelium (r²=0.73) and not with intimal medial index (r²=0.47). The correlation between amount of plaque, to loss of endothelium, intimal medial index, and maximal relaxation to calcium ionophore A23187 was less significant (r²=0.42, 0.46, 0.42, respectively). These data suggest that the defect in endothelium-mediated relaxation is primarily due to the destruction of endothelial cells in vessels with severe atherosclerosis, but dysfunction of endothelium may also play a role in impaired relaxation. Endothelium-dependent relaxation appeared to decrease with plaque progression, which may be related to hyperlipidemia and/or atherosclerosis in vessels with mild focal endothelial cell disruption. However, no statistically significant differences were noted between groups 1 and 2. Increased sensitivity to histamine and serotonin in WHHL rabbits cannot be accounted for by the loss of endothelial cells during atherosclerotic progression because no differences were seen in denuded vessels.

Other factors in addition to endothelial cell loss, such as activation of resident leukocytes, may contribute to alterations in vascular reactivity. Accumulation of monocytes/macrophages is a common occurrence in atherosclerotic lesions, and recent evidence

<table>
<thead>
<tr>
<th>Table 4. Morphological Evaluation of Vascular Ring Segments in Control and Watanabe Heritable Hyperlipidemic Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control (n=5)</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>Plaque (%)</td>
</tr>
<tr>
<td>Endothelium loss (%)</td>
</tr>
<tr>
<td>I/M index</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Control, New Zealand White rabbits; group 1, Watanabe heritable hyperlipidemic (WHHL) rabbits aged 3–5 months; group 2, WHHL rabbits aged 6–9 months; group 3, WHHL rabbits aged 12–14 months; AA, aortic arch; TA, thoracic aorta; I/M, intima/media area times 100.

*p<0.05 compared with AA in control and in groups 1 and 2.
suggestions that a vasoactive product released by these cells may contribute to vasospasm in atherosclerosis. Infusion of fmet-leu-phe (a known activator of monoocytes/macrophages) into perfused hindlimbs of atherosclerotic monkeys produces an increase in vascular resistance when compared with that in nonatherosclerotic animals. This vasoconstrictor response may selectively involve release of prostaglandin E_2.

Infusion of leukotriene D_4 had minimal effects in both atherosclerotic and nonatherosclerotic animals, whereas prostaglandin E_2 produced a greater than 10-fold increase in atherosclerotic vessels. Other endogenous products of monocytes/macrophages, such as superoxide anions, may also contribute to altered vascular tone. Release of superoxide anions (in vitro) depresses endothelium-mediated relaxations to acetylcholine.

Previous Studies

Contractile responses. Contraction elicited by phenylephrine is caused by activation of α_1-adrenergic receptors located on smooth muscle cells. In the present study, the contractile responses to phenylephrine are decreased in aortas obtained from WHHL rabbits with moderate and severe atherosclerosis as compared with control vessels. This reduction, however, was not reflected by a significantly decreased ED_{50} value. These results are different from previous reports in cholesterol-fed and WHHL rabbits in which contractile responsiveness to the adrenergic agonist phenylephrine was unaltered.

In rabbits fed a cholesterol-rich diet, an increased number of α-adrenergic receptors have been detected. Despite the increase in receptor number, the results of our study and previous studies in cholesterol-fed and WHHL rabbits do not demonstrate augmented contraction to phenylephrine.

Several studies have described augmented constrictor responses to serotonin in animal models of atherosclerosis. Yokoyama et al have reported an increased responsiveness to serotonin in atherosclerotic aortas from WHHL rabbits aged 8–12 months. More recently, they observed augmented contraction to serotonin in ring segments of WHHL rabbits aged 10–22 months with moderate atherosclerosis compared with ring segments of rabbits with mild or severe atherosclerosis. We have demonstrated that vasoconstrictor responses elicited by serotonin were markedly increased in aortas only during the early stages of hypercholesterolemia and/or atherosclerosis. Augmented constrictor responses to serotonin have been demonstrated in aortas from rabbits fed a 2% and 0.3% cholesterol-rich diet for 10 weeks and 16 weeks, respectively, and in cholesterol-fed cynomolgus monkeys. Hypersensitivity to serotonin in atherosclerotic vessels may be due to an increased number of serotoninergic receptors. Results from the present...
study suggest that an increase in serotonin receptor density is an early phenomenon in atherosclerosis.

Histamine causes vasoconstriction as a result of stimulating the H₁-receptor subtype.³¹ To our knowledge, there are no previous vascular reactivity studies evaluating the effect of histamine on atherosclerotic vessels in the rabbit model although an increased sensitivity to histamine has been reported in atherosclerotic vessels from miniature pigs and human coronary arteries.¹⁸,¹⁹,³² Vascular constriction in the aortas of all groups of WHHL rabbits showed an enhanced responsiveness to histamine. Augmented constrictor responses to histamine may be due to an increase in H₁-receptor density as previously described in aortas of rabbits that were fed cholesterol.³³ Other mechanisms may involve increased sensitivity of the receptor and/or effector units after stimulation by histamine in the presence of atherosclerosis.¹⁸

In the present study, contractile responses to phenylephrine, histamine, and serotonin were unchanged by mechanical removal of endothelium. Increased contractile responses to these agents have been reported in denuded vessels of rat, dog, and swine.³⁴-³⁶ However, Furchgott⁶ and Verbeuren et al.¹² have demonstrated that removal of endothelial cells in the rabbit aorta does not cause enhanced vasoconstriction. Therefore, augmented contractions to histamine and serotonin are most likely due to alterations in smooth muscle cell functions rather than loss of endothelial cells in this model. Previous studies demonstrate that vasoconstrictor responses to KCl in normal and atherosclerotic vessels are similar; therefore, it appears that the contractile machinery in atherosclerotic vessels is not altered.¹⁵,²⁷

In the present study, vasoconstrictor responses to serotonin were augmented in vessels with mild atherosclerosis, whereas hyperresponsiveness to hista-
mine was noted independent of the severity of the lesion. Ginsburg et al. demonstrated that the pharmacological sensitivity of certain vasoconstrictor substances differs in the ability to mediate contraction and is dependent on calcium pools. They showed that coronary arteries use both "intracellular" and "extracellular" calcium in response to various vasoconstrictor agents and that the use of these calcium pools is specific for each receptor pathway. Increases in cholesterol content of membranes in early atherosclerosis may cause an increased permeability to calcium. In contrast to histamine, contractile responses to serotonin in human coronary arteries are more dependent on extracellular calcium. Therefore, if calcium permeability is reduced in later stages of atherosclerosis, contractile responses to serotonin may be decreased.

Relaxation responses. Numerous studies have confirmed that vascular endothelium selectively modulates smooth muscle contraction indirectly by the release of an endothelium-derived relaxing factor. Endothelium-derived relaxing factor, now thought to be nitric oxide, is suggested to have a mechanism of action on vascular smooth muscle similar to nitroglycerin. Both compounds activate soluble guanylate cyclase, which leads to increased levels of cyclic GMP and subsequent relaxation. In addition to promoting vasodilation, the endothelium attenuates vasoconstrictor effects of several agents, such as platelets, serotonin, and norepinephrine. It is speculated that impairment or loss of endothelial cell function may predispose atherosclerotic vessels to vasospasm.

Several recent studies indicate that cholesterol feeding impairs endothelium-dependent relaxation in New Zealand White rabbits. Verbeuren et al. have shown that endothelium-dependent relaxations to acetylcholine and ATP are impaired in rabbits as early as 8 weeks after diet-induced hypercholesterolemia (0.3% cholesterol). In addition, impairment of endothelium-dependent relaxation

![Figure 7. Scanning electron micrograph of the endothelial surface from the aortic arch of a group 1 rabbit showing monocytes focally adhering to the endothelium (panel A) and a high power view of a focal collection of monocytes (panel B).](image-url)
correlated with the degree of atherosclerotic plaque. Similarly, Jayakody et al\textsuperscript{42} have reported that feeding rabbits a diet high in cholesterol (2\%) (cholesterol levels $>2,000$ mg/dl) for 4 weeks produced fatty streaks in the aorta and that these were associated with morphological alterations in endothelial cells, which correlated with a decrease in endothelium-dependent relaxation. Fatty streak lesions had swollen endothelial cells with numerous cytoplasmic projections and early occasional irregularly shaped ulcerations. Yokoyama et al\textsuperscript{29} in a recent preliminary report, described progressive attenuation of endothelium-dependent relaxation in three age groups of WHHL rabbits: less than 9 months, 10–22 months, and more than 23 months. However, a description of endothelial cell morphology was lacking; thus, comparison with the present study was difficult.\textsuperscript{29}

Impairment of endothelium-dependent relaxation in diet-induced hypercholesterolemia may be specific for relaxations mediated by receptors. Bossaller et al\textsuperscript{44} have observed that other agents, in particular the calcium ionophore A23187, which elicits responses independent of receptors, were effective in relaxing the coronary artery and aorta obtained from cholesterol-fed rabbits but that responses to acetylcholine were significantly reduced. Similar findings have been reported in atherosclerotic vessels removed from cholesterol-fed pigs, cynomolgus monkeys, and humans with atherosclerotic coronary arteries.\textsuperscript{16,17,32,45} These data suggest that in atherosclerotic vessels there are selective alterations of muscarinic receptors and that other features of endothelium-dependent relaxation are preserved.

Our studies of WHHL rabbits with hyperlipidemia and/or mild atherosclerosis did not demonstrate
impairment of endothelium-dependent relaxation. In preparations of mild to moderate atherosclerotic tissues with primarily intact endothelium (12±4%), relaxation responses to acetylcholine, ATP, and calcium ionophore A23187 were relatively maintained. However, in tissues with severe atherosclerosis (36±4%), the loss of endothelial cells resulted in significant attenuation of endothelium-dependent relaxation in response to these agents. One might argue that because blockers of prostaglandin synthesis were not present in our studies, maintenance of endothelium-dependent relaxation in vessels with early lesions may be the result of endogenous prostacyclin released from endothelial cells and was independent of EDRF mechanisms. However, other studies in normal and atherosclerotic vessels removed from cholesterol-fed rabbits have demonstrated a minimal role of prostacyclin in promoting vascular relaxation elicited by acetylcholine.42,46 In addition, prostacyclin (prostaglandin I2) is suppressed in aortas from cholesterol-fed rabbits.47

One possible explanation for the preservation of endothelium-dependent relaxation in vessels with mild and moderate atherosclerosis is that WHHL rabbits are hyperlipidemic from birth. It is conceivable that endothelial cells from these animals, which were predominantly intact, may have adapted to a high cholesterol environment and, therefore, function normally as opposed to animals with acute diet-induced hypercholesterolemia. In addition, endothelium-dependent responses to calcium ionophore A23187 were similarly attenuated in vessels with severe atherosclerosis in which there was a substantial loss of endothelial cells. We believe that destruction of endothelial cells may be the dominant mechanism of impaired vasodilation in the WHHL rabbit model. However, endothelial cell dysfunction cannot be excluded.
Impaired relaxation to acetylcholine in aortas from spontaneously hypertensive rats has been shown, and this abnormality is associated with a concomitant production of an endothelium-derived constricting factor.\textsuperscript{48} In the present study, in atherosclerotic and control vessels with and without endothelium, augmented vasoconstriction to acetylcholine could not be demonstrated (data not shown). Therefore, a decreased endothelium-derived relaxing factor response to acetylcholine was not related to concomitant production of an endothelium-derived constricting factor by vessels with severe atherosclerosis or enhanced activation of muscarinic receptors in smooth muscle cells of atherosclerotic vessels.

In summary, vasoconstrictor responses to the \(\alpha\)-agonist phenylephrine progressively decreased with increasing plaque formation, whereas responses to histamine and serotonin were augmented by hyperlipidemia and/or atherosclerosis. Increased sensitivity to histamine and serotonin was independent of endothelial cell function in that removal of endothelial cells did not potentiate vasoconstriction. Thus, selective alteration(s) in smooth muscle cell function in atherogenic vessels rather than loss of endothelial cells may precipitate hypersensitivity to histamine and serotonin in the WHHL rabbit model.

Endothelium-mediated relaxation in the WHHL rabbit is attenuated with atherosclerotic progression. The mechanism of this defect in the WHHL rabbit model appears to be due to a significant loss of endothelial cells in plaque areas as opposed to alterations in endothelial cell function and/or mechanisms subsequent to the release of endothelium-derived relaxing factor. Loss of endothelial cells has been speculated to be of importance in atherosclerosis, and the findings of our study confirm this hypothesis.

**FIGURE 10.** Scanning electron micrograph of the edge of a fibrous plaque from a group 3 Watanabe heritable hyperlipidemic rabbit showing V-shaped loss of endothelium (arrows) (panel A) and a high power view of the border areas of a fibrous plaque (arrows) (panel B).
FIGURE 11. Scanning electron micrograph showing intimal surface from a group 3 Watanabe heritable hyperlipidemic rabbit with total destruction of the endothelium in an animal that had undergone mechanical denudation (rubbed segment). Only subendothelial fibers are seen.

References


40. Holzmann S: Endothelium-induced relaxation by acetylcholine is associated with larger rises in cyclic GMP in coronary arterial strips. J Cyclic Nucleotide Protein Phosphor Res 1982;8:409–419


46. Forstermann U, Henting G, Neufang B: The role of endothelium and non-endothelial prostaglandins in the relaxation of isolated blood vessels of the rabbit induced by acetylcholine and bradykinin. Br J Pharmacol 1986;87:521–532


Key Words: atherosclerosis, endothelium-derived relaxing factor, endothelium, vascular reactivity, rabbits
Vascular reactivity during the progression of atherosclerotic plaque. A study in Watanabe heritable hyperlipidemic rabbits.
F D Kolodgie, R Virmani, H E Rice and W J Mergner

Circ Res. 1990;66:1112-1126
doi: 10.1161/01.RES.66.4.1112

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/66/4/1112

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/