Impaired Vasodilatory Response to Atrial Natriuretic Peptide During Atherosclerosis Progression

Ken-ichi Hirata, Hozuka Akita, Mitsuhiro Yokoyama, and Yoshio Watanabe

This study was undertaken to examine the alterations in vascular relaxation responsiveness to endothelium-dependent or -independent vasodilators, including atrial natriuretic peptide (ANP) and acetylcholine, in aortas of Watanabe heritable hyperlipidemic (WHHL) rabbits during the progression of the atherosclerotic plaque. WHHL rabbits were divided into two groups according to age: group 1, 6–11 months, and group 2, 12–18 months. The isolated thoracic aortas obtained from both normal (control) and WHHL rabbits were suspended in a bath containing oxygenated Krebs' buffer for recording of isometric force. The endothelium-dependent relaxation evoked by acetylcholine was reduced in group 1 WHHL rabbits and decreased progressively in proportion to the degree of atherosclerosis progression when compared with age-matched control rabbits. ANP-induced relaxation was not significantly decreased in group 1 WHHL rabbits. However, ANP-induced relaxation was markedly impaired in group 2 WHHL rabbits. Thoracic aortas with severe atherosclerosis were less sensitive to ANP, with a significant increase in the median effective dose, although maximum relaxation induced by ANP was not reduced. Accumulation of cyclic GMP induced by ANP and acetylcholine was markedly reduced in atherosclerotic arteries obtained from group 2 WHHL rabbits compared with control rabbits. Vascular relaxation elicited by nitroglycerin or isoproterenol was not significantly impaired in atherosclerotic arteries from either group 1 or group 2 WHHL rabbits. From these results, we suggest that ANP-induced cyclic GMP formation and vascular relaxation via particulate guanylate cyclase in vascular smooth muscle cells are impaired in severely atherosclerotic arteries. (Arteriosclerosis and Thrombosis 1992;12:99-105)

Coronary spasm is now recognized as an important cause of myocardial ischemia in patients with ischemic heart disease, including variant angina, unstable angina, and acute myocardial infarction, but the mechanisms responsible for the occurrence of spasm remain unclear. Coronary spasm usually occurs at the site of atherosclerotic lesions of varying severity. There is accumulating evidence that atherosclerotic vessels from experimental animals and humans exhibit enhanced susceptibility to the constrictor effects of ergonovine, histamine, and serotonin and that they show depressed responsiveness to endothelium-dependent vasodilators including acetylcholine and substance P. These alterations of vascular reactivity to vasoconstrictor and vasodilator stimuli in the atherosclerotic coronary artery may play a significant role in the pathogenesis of coronary spasm.

Since the discovery of an atrial natriuretic factor in 1981, there have been numerous studies documenting the vasodilator properties of atrial natriuretic peptides (ANPs) extracted from the human heart and of synthetic atrial peptides in isolated vessels obtained from various experimental animals and humans. Guanosine-3',5'-cyclic monophosphate (cyclic GMP) has been reported to be elevated in response to a variety of vasodilators including ANP, nitroglycerin, and endothelium-derived relaxing factor(s) (EDRF), and the increase in cyclic GMP levels noted in isolated blood vessels parallels the magnitude of the relaxation response of the vessels to the vasodilators. The present study was designed to determine whether impaired relaxation by ANP and endothelium-dependent relaxation induced by acetylcholine are demonstrable during atherosclerosis.
progression in Watanabe heritable hyperlipidemic (WHHL) rabbits and to clarify the mechanism of impairment of ANP-induced relaxation.

Methods
Animal Model of Atherosclerosis
WHHL rabbits of either sex were used in these experiments. These animals exhibit consistent hereditary hyperlipidemia as a result of inbreeding, and their vascular lesions are morphologically comparable with those of human atherosclerosis. The atherosclerotic changes always appear by 6 months and progress with advancing age. WHHL rabbits aged 6–11 months (group 1, n=6) and 12–18 months (group 2, n=10) were examined in this study. Age-matched normal Japanese white rabbits (n-18) were studied as controls. All animals were fed standard rabbit chow (Orientalkobo, Tokyo, Japan).

Morphological Study
After the end of each experiment, the vascular specimens were photographed, and then the total surface and atheromatous plaque areas were quantified by planimetry.

Pharmacological Measurements
The rabbits were anesthetized with pentobarbital sodium (30 mg/kg body wt i.v.), and the descending thoracic aortas were isolated and cleaned of surrounding tissue. Aortic rings approximately 3-mm wide were cut and opened. For recording of isometric force, transverse aortic strips were suspended in 30-ml organ baths containing a buffer of the following composition (values in mM): NaCl 118, KCl 4.0, CaCl\(_2\) 1.5, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, and glucose 5.8 and equilibrated at 37°C with a 95% O\(_2\)-5% CO\(_2\) gas mixture. Final pH was approximately 7.38. One end of the strip was attached to the bottom of the chamber, and the other end was attached to a Statham 4C-2 force transducer (Gould, Inc., Glen Burnie, Md.), which was connected to a Nihonkoden amplifier/recorder system (Nihonkoden, Tokyo, Japan). An initial preload of 1.5 g was applied, and the strips were allowed to stabilize for 2 hours. A test contraction was induced by raising the KCl concentration to 20 mM. When developed tension attained its peak value, the strips were relaxed by rinsing with the buffer. Then the strips were contracted with 0.3 \(\mu\)M phenylephrine and then subsequently relaxed by the cumulative addition of human ANP (0.01 nM–1 \(\mu\)M), acetylcholine (0.01–1 \(\mu\)M), nitroglycerin (1 nM–1 \(\mu\)M), or isoproterenol (1 nM–1 \(\mu\)M). In some experiments, the endothelium was removed mechanically by rubbing the intimal surface with filter paper moistened with the buffer. Relaxation values were expressed as percent decreases of the phenylephrine (0.3 \(\mu\)M)-induced constrictor tone.

Measurements of Cyclic GMP and Cyclic AMP
Aortic rings approximately 3–4-mm wide were used for the measurements of cyclic GMP and cyclic AMP. Aortic segments about 9–12-mm long were cut into three rings of equal width. The two outer rings were used for mechanical experiments as described above, and the middle ring was used for the measurement of cyclic GMP and cyclic AMP. Aortic rings with endothelial denudation were used for formation of cyclic nucleotides after stimulation with ANP, nitroglycerin, and isoproterenol, which elicited endothelium-independent relaxation, because ANP causes cyclic GMP formation in endothelial cells as previously described. On the other hand, aortic rings with intact endothelium were used for the measurement of cyclic GMP levels after stimulation with acetylcholine, an endothelium-dependent vasodilator. After incubation with oxygenated Krebs' buffer for 60 minutes, selected concentrations of drugs in Krebs' buffer were added. At selected intervals after the drugs were added, the strips were freeze-clamped with a Wollenberger clamp (Natsumeseikakusho, Tokyo, Japan) in liquid nitrogen. The tissue was subsequently homogenized in a glass–glass homogenizer in ice-cold 6% trichloroacetic acid. The homogenates were centrifuged at 1,700g for 5 minutes at 4°C. Precipitates were used for protein determination by the method of Bradford with bovine serum albumin as the standard. Supernatant fractions were extracted three times with ether, and cyclic GMP and cyclic AMP were measured in duplicate by radioimmunoassay (Yamasashoyou, Choshi, Japan).

Drugs
The following drugs were used: 1-phenylephrine hydrochloride, acetylcholine chloride, isoproterenol hydrochloride (Sigma Chemical Co., St. Louis, Mo.), human ANP (Peptide Institution, Osaka, Japan), and nitroglycerin (Nihonkayaku, Tokyo, Japan). The drugs were dissolved in distilled water and then diluted in buffer. All concentrations are expressed as final concentrations.

Data Analysis and Statistics
Data are expressed as mean±SEM. Relaxation response is expressed as the percent relaxation from the amount of precontraction produced by 0.3 \(\mu\)M phenylephrine. The significance of the difference between group means was analyzed by one-way analysis of variance and the Bonferroni test for samples. Probability values <0.05 were taken as statistically significant.

Results
Serum Cholesterol and Triglyceride Levels
Body weights and serum cholesterol and triglyceride levels are shown in Table 1. Total serum cholesterol and triglyceride levels were markedly elevated in WHHL rabbits compared with control rabbits.
### Table 1. Experimental Animal Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>WHHL Group 1</th>
<th>WHHL Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (kg)</td>
<td>3.2±0.1</td>
<td>3.2±0.1</td>
<td>3.4±0.1†</td>
</tr>
<tr>
<td>Chol (mg/dl)</td>
<td>45±6</td>
<td>468±90*</td>
<td>432±80*</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>43±20</td>
<td>330±93*</td>
<td>318±68*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM.

Control, Japanese white rabbits; WHHL, Watanabe heritable hyperlipidemic rabbits; group 1, WHHL rabbits aged 6–11 months; group 2, WHHL rabbits aged 12–18 months; BW, body weight; Chol, serum cholesterol; TG, serum triglyceride.

Significantly different from the control group: *p<0.001, †p<0.01.

### Morphological Study

No visible atherosclerotic lesions were detected in any vessels removed from Japanese white rabbits. Macroscopic study of vessels from WHHL rabbits indicated that all the segments of the descending thoracic aortas used in the force measurement determinations had grossly visible dots of lipid deposition. Atherosclerotic lesion areas as percentages of the total surface area in the descending thoracic aortas in group 1 and 2 WHHL rabbits were 52±11% and 92±7%, respectively.

### Pharmacological Study

In thoracic aortas obtained from control rabbits, ANP caused dose-dependent relaxation, and endothelial removal had no effect on ANP-induced relaxation in normal rabbits (Figure 1). Endothelial denudation was confirmed by the absence of relaxation induced by acetylcholine. These results indicated that ANP-induced relaxation was endothelium independent. There were no statistically significant differences in any vascular relaxations induced by ANP, acetylcholine, isoproterenol, or nitroglycerine between the 6–11-month-old and the 12–18-month-old Japanese white rabbits (data not shown). Therefore, these vasodilator responses were not altered by aging. Isometric contractions induced by phenylephrine or 20 mM KCl were almost identical in group 1 and group 2 WHHL rabbits and control rabbits (data not shown). A representative recording of ANP-induced relaxation in aortic strips obtained from group 1 and group 2 WHHL and normal rabbits is demonstrated in Figure 2. The vasodilator response to ANP was markedly attenuated compared with that for control rabbits, and the concentration–relaxation relations for ANP in group 2 WHHL rabbits were markedly shifted to the right compared with control and group 1 WHHL rabbits (Figure 3 and Table 2). However, at maximal doses, ANP was able to elicit complete relaxation of all the blood vessels tested. The vasodilator response of aortas obtained from group 1 WHHL rabbits to acetylcholine, which produces EDRF(s), was remarkably attenuated compared with that of control rabbits, and acetylcholine-induced relaxation was almost abolished in group 2 WHHL rabbits (Figure 4 and Table 2). Concentration–response relations for nitroglycerin and isoproterenol were almost identical among the three groups of rabbits (Figures 5 and 6 and Table 2).

![Figure 1](https://example.com/f1.png)

**Figure 1.** Relaxation response curves to human atrial natriuretic peptide (ANP) in control rabbit aortas with (●) or without (○) endothelium. X-axis values are final concentrations of ANP (−log M) in the organ bath, and y-axis values are percent relaxation. Each point is the mean of 12 observations. Vertical bars indicate SEM.

![Figure 2](https://example.com/f2.png)

**Figure 2.** Typical response curves to atrial natriuretic peptide in control (panel A), group 1 WHHL (panel B), and group 2 WHHL (panel C) rabbit aortas. Concentrations of all drugs are expressed as negative logarithms. WHHL, Watanabe heritable hyperlipidemic; PE, phenylephrine (0.3 μM).
Formation of Cyclic Nucleotides

Table 3 shows the results of the measurements of cyclic GMP after stimulation with acetylcholine, ANP, or nitroglycerin. Before stimulation, cyclic GMP levels did not differ significantly between aortic strips of control rabbits and those of WHHL rabbits. In both groups, the maximal increase in cyclic GMP levels after stimulation with the three drugs corresponded to the 1-minute values. On the basis of these time-course observations, 1-minute values were taken to represent peak increases in cyclic GMP.

Endothelial removal elicited decreased cyclic GMP levels in control rabbits. Although the increases in cyclic GMP levels in both groups were similar for nitroglycerin, they differed for acetylcholine or ANP, with the severely atherosclerotic arteries of group 2 WHHL rabbits exhibiting significantly lower values than the nonsclerotic arteries of control rabbits (Table 2). On the other hand, the formation of cyclic AMP stimulated by 1 μM isoproterenol in aortic strips from group 2 WHHL rabbits was almost identical to that from control rabbits. Increases in cyclic AMP levels elicited by isoproterenol (1 μM) were 12.7±3.9 and 10.9±4.1 nM/mg protein in group 2 WHHL rabbits and control rabbits, respectively.

Discussion

The present study provides the first documentation that endothelium-independent relaxation and cyclic GMP formation induced by ANP are depressed in atherosclerotic arteries. In moderately atherosclerotic arteries, endothelium-dependent relaxation by acetylcholine was decreased and was progressively attenuated during atherosclerotic progression. In severely atherosclerotic arteries, vascular relaxation evoked by ANP was markedly reduced compared with that in control rabbits, although relaxation by nitroglycerin or isoproterenol was well preserved.

From age-matched control rabbit experiments, vascular relaxation elicited by ANP was not altered by aging. Therefore, a decreased relaxation response to ANP was strongly related to the extent of atherosclerotic lesions.

ANP is a potent bioactive peptide that possesses natriuretic,22 vasorelaxant,23 and aldosterone-inhibiting24 properties. Numerous studies have shown that ANP elicits relaxation of isolated vessels obtained from experimental animals. Furthermore, ANP causes vasodilation in epicardial coronary arteries in vivo,25-27 and ANP is thought to modulate coronary blood flow. It is well documented that ANP-evoked relaxation is mediated by the accumulation of cyclic GMP levels generated through the stimulation of ANP receptors coupled to particulate guanylate cyclase in vascular smooth muscle cells. On the other hand, endothelium-derived nitric oxide and nitrovasodilators (including nitroglycerin) elicit cyclic GMP formation through the stimulation of soluble (cytosolic) guanylate cyclase in vascular smooth muscle cells. In the present study, nitroglycerin-induced cyclic GMP formation and vascular relaxation were well preserved in severely atherosclerotic arteries from group 2 WHHL rabbits compared with those of control rabbits. However, cyclic GMP formation stimulated by ANP was significantly reduced in severely atherosclerotic arteries. From these results, impairment of ANP-induced relaxation in severely atherosclerotic arteries is thought to have been caused by the attenuation of cyclic GMP formation via particulate guanylate cyclase in vascular smooth muscle cells.

Table 2. Relaxation Response to Vasodilators in Aortic Strips From Control and Watanabe Heritable Hyperlipidemic Rabbits

<table>
<thead>
<tr>
<th>Animal group</th>
<th>ANP</th>
<th>ACh</th>
<th>NTG</th>
<th>Isop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1, WHHL</td>
<td></td>
<td></td>
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<tr>
<td>Group 2, WHHL</td>
<td></td>
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</tbody>
</table>

Values are expressed as mean±SEM.

ANP, atrial natriuretic peptide; ACh, acetylcholine; NTG, nitroglycerin; Isop, isoproterenol; ED50, dose to induce 50% relaxation; Max, maximum relaxation (percent of phenylephrine-induced contraction); group 1 WHHL, aged 6–11 months; group 2 WHHL, aged 12–18 months.

*p<0.001 vs. controls.
The mechanisms of impairment of cyclic GMP formation in severely atherosclerotic arteries are unknown. Although the concentration–response curve to ANP was shifted to the right and the median effective dose values were increased in severely atherosclerotic arteries, its maximum relaxation was not reduced. This observation suggests that the impairment of ANP-induced relaxation may be caused by alteration of ANP receptors.

Recently, subclasses of ANP receptors have been investigated. Two cell-surface receptors for ANP have been described: a low-molecular-weight “clearance” receptor (C-receptor) and a high-molecular-weight, cyclic GMP-coupled receptor (the biologically active, or B-receptor). ANP elicits cyclic GMP formation via the B-receptor, and ANP also binds to the C-receptor, which is regarded to serve in the clearance of ANP. In severely atherosclerotic arteries, alterations in the number or affinity of binding sites for ANP or the distribution of B- and C-receptors may reduce the increases in cyclic GMP levels stimulated by ANP. Plasma ANP levels in some group 2 WHHL rabbits were elevated fivefold to 10-fold higher than in those of control rabbits (M. Yokoyama et al, unpublished observation). Therefore, we cannot exclude the possibility that the desensitization of ANP receptors caused the impairment of ANP-induced relaxation in severely atherosclerotic arteries.

Several reports demonstrate that impaired endothelium-dependent relaxation may play an important role in the altered regulation of vascular tone in atherosclerotic arteries. In our experiments, ANP-induced endothelium-independent relaxation through particulate guanylate cyclase was also impaired in severely atherosclerotic arteries. From these results, dysfunction of smooth muscle cells for specific agonists including ANP, as well as that of endothelial cells, may cause altered vascular regulation in atherosclerotic arteries.

Endothelium-dependent relaxation induced by acetylcholine was impaired in moderately atherosclerotic arteries and decreased during the progression of atherosclerotic plaque formation. The impairment of EDRF-mediated relaxation was observed at an earlier stage of atherosclerosis compared with that of ANP-induced relaxation. Numerous studies have confirmed that the vascular endothelium indirectly modulates smooth muscle tone by the release of cyclic GMP.
EDRF(s). There are several reports that demonstrate that endothelium-dependent relaxation is impaired in atherosclerotic arteries obtained from animals and humans. The following explanations are proposed for the impairment of endothelium-dependent relaxation in atherosclerotic arteries. First, the production and/or release of EDRF may be decreased. Second, the thickened intima may reduce the diffusion of EDRF because the half-life of EDRF is extremely short. Third, the characteristics of smooth muscle cells and, in particular, their sensitivity to EDRF may be changed. This possibility is unlikely because the relaxation to nitroglycerin, which induces relaxation through activation of guanylate cyclase in smooth muscle cells as does EDRF, was well maintained in severely atherosclerotic arteries. Recently, Kolodgie et al have demonstrated that endothelium-mediated relaxation is reduced in WHHL rabbits because of a loss of endothelial cells. At present, precise mechanisms for the attenuation of EDRF-mediated relaxation in atherosclerotic arteries are still debated.

In summary, ANP-induced relaxation was impaired due to decreased cyclic GMP formation via particulate guanylate cyclase of smooth muscle cells in severely atherosclerotic arteries. Moreover, endothelium-dependent relaxation was impaired at an earlier stage of atherosclerosis compared with the ANP-elicited relaxation. These alterations in vascular responsiveness to some vasodilators may contribute to the pathophysiological regulation of coronary circulation in atherosclerotic ischemic heart disease.

Acknowledgment

The authors wish to thank Noriko Hamana for her skillful technical assistance.

References


KEY WORDS • atherosclerosis • atrial natriuretic peptide • endothelium-derived relaxing factor • cyclic GMP • Watanabe heritable hyperlipidemic rabbits
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doi: 10.1161/01.ATV.12.1.99

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