Augmented Vasoconstrictor Responses to Serotonin Precede Development of Atherosclerosis in Aorta of WHHL Rabbit

Phillip A. Wines, James M. Schmitz, Sandra L. Pfister, Fred J. Clubb, Jr., L. Maximilian Buja, James T. Willerson, and William B. Campbell

Watanabe heritable hyperlipidemic (WHHL) rabbits have elevated concentrations of plasma cholesterol and develop progressive atherosclerosis. The present investigation was undertaken to evaluate the vascular responses to vasoactive compounds of aortas from WHHL rabbits and normal New Zealand Whites (NZW) rabbits at 1 and 6 months of age. Rings of distal thoracic aortas were suspended under isometric tension in oxygenated Krebs buffer. Developed tension was measured in response to graded concentrations of agonists. Maximal responses to KCl (40 mM) were the same in aortas from the 1-month-old and 6-month-old WHHL and NZW rabbits. Aortas from 1-month-old animals were more sensitive to serotonin than aortas from 6-month-old animals. Aortas from WHHL rabbits exhibited an increased maximal response to serotonin when compared with NZW controls. In contrast, the constrictor responses to norepinephrine were reduced in WHHL rabbits compared with NZW rabbits at both age groups. Methacholine decreased tension development in serotonin-contracted vessels. This relaxation was greatest in aortas from NZW rabbits. In 1-month-old NZW rabbits fed a high cholesterol diet, the constrictor responses to serotonin and the relaxation responses to methacholine did not differ from NZW rabbits ingesting a normal diet. However, the responses to norepinephrine were markedly attenuated in the hypercholesterolemic NZW rabbits. Microscopic evaluation of the aortas revealed occasional adherent leukocytes and irregularities in the vascular endothelium in 1-month-old WHHL animals. These changes were greater in aortas from 6-month-old WHHL animals, with more adherent leukocytes, adherent platelets, and severe irregularities in the endothelial surface. These results indicate that the vasoconstrictor responses to serotonin are augmented in WHHL rabbits, and this enhanced response precedes the development of gross, but not microscopic, atherosclerotic changes in the blood vessel. The enhancement of vasoconstrictor responses to serotonin in the WHHL rabbit cannot be attributed to hypercholesterolemia. (Arteriosclerosis 9:195–202, March/April 1989)

Coronary artery vasospasm has been proposed as one mechanism to account for the episodic reductions in myocardial oxygen delivery in patients with unstable angina pectoris. Atherosclerosis may contribute to the development of coronary artery vasospasm through several mechanisms. Atherosclerosis may result in altered responses of the epicardial coronary vessels to vasoactive stimuli by either augmenting responses to vasoconstrictors or diminishing the production of compensatory vasodilatory substances, such as prostacyclin. Also, atherosclerosis may promote the local accumulation of platelets, neutrophils, or macrophages. These cells may release vasoconstrictors that may promote vascular spasm. Other investigators have suggested that hypercholesterolemia per se may augment the reactivity of blood vessels. In the present investigation, we hypothesized that augmented vasoconstrictor responses occur in blood vessels obtained from hypercholesterolemic animals that are prone to develop atherosclerosis.

In these studies, we used the Watanabe heritable hyperlipidemic (WHHL) rabbit. Homozygous WHHL rabbits produced by serial inbreeding have plasma cholesterol concentrations of 500 to 950 mg/dl, develop grossly visible aortic atherosclerosis by 5 months of age, and have decreased numbers of functioning low density lipoprotein (LDL) receptors in several tissues. Thus, this animal model exhibits many clinical and biochemical similarities to familial hypercholesterolemia. To test our hypothesis, we evaluated the vasoconstrictor responses of isolated segments of thoracic aorta obtained from normal and WHHL rabbits in two different age groups. Our results demonstrate an increased sensitivity to serotonin that precedes the development of established atherosclerotic lesions of the aorta.

From the Departments of Pharmacology, Internal Medicine (Cardiology), and Pathology, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas.

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Address for correspondence: William B. Campbell, Ph.D., Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235.

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Methods

New Zealand White (NZW) rabbits were used as controls. WHHL rabbits were bred from a mating pair of homozygous WHHL rabbits obtained from Dr. Yoshio Watanoabe and provided to us by Michael Brown and Joseph Goldstein at our institution.7-12 The animals were individually caged and were fed Purina Lab Rabbit Chow. NZW or WHHL rabbits of either sex were sacrificed by cervical dislocation at 4 to 5 weeks of age (1 month) or at 5 to 6 months of age (6 months). The distal thoracic aorta was carefully removed and placed into modified Krebs bicarbonate buffer as follows: 118 mM NaCl, 4.0 mM KCl, 3.3 mM CaCl₂, 24 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 11 mM glucose. Loose adventitial tissue was carefully dissected from the vessels. Then the vessels were sliced into 2 mm thick segments using care to avoid disrupting the endothelium. Aortic rings were suspended in 30 ml tissue baths containing Krebs-bicarbonate buffer maintained at 37°C and continuously bubbled with 95% O₂/5% CO₂. Isometric tension was measured by using force displacement transducers (Grass, Quincy, MA) and continuously recorded using a Grass Polygraph. The optimal resting tension in aortic rings from 1- and 6-month-old WHHL and NZW rabbits was 2.0 g. This value was determined by increasing the length of the rings in a stepwise fashion and measuring the active tension generated by exposing the rings to 20 mM KCl. The rings were allowed to equilibrate at resting tension for 1 hour. Maximal contractions were produced by raising the KCl concentration of the tissue baths to 40 mM. After attaining peak contractions, the rings were allowed to relax by replacing the bath solution with one buffer containing 4 mM KCl. Test contractions were repeated in this manner until peak contractions attained a reproducible maximum.

Concentration-response curves were obtained by the cumulative addition of either serotonin, norepinephrine, histamine, or the thromboxane mimetic, U46619. After maximal contractions to any given agent were attained, the tissue baths were rinsed with buffer, and the tissue was allowed to return to resting tension. Test contractions were repeated with 40 mM KCl, and the tissue was allowed to stabilize before concentration-response curves to additional agents were performed. To alleviate any potential effect of either the duration of the experiments or the order in which the agents were studied, the drugs were given in random order, differing from experiment to experiment. Endothelial integrity was demonstrated at the conclusion of each experiment by precontracting the rings with serotonin (10⁻⁶M) and determining the concentration-dependent relaxation responses to methacholine. Responses to methacholine obtained at the conclusion of an experiment did not differ significantly from those observed before the addition of any other agents. While serotonin reportedly causes endothelium-dependent relaxation of the canine coronary artery,13 this does not appear to occur in the rabbit aorta. When the effects of serotonin on intact and de-endothelialized aortic rings were compared, serotonin-induced contractions were identical in the two groups, and no relaxation was observed (data not shown).

In an additional set of experiments, 1-month-old NZW rabbits were placed on a diet containing 2% cholesterol (ICN Biochemicals, Cleveland, OH) for 2 weeks. Plasma cholesterol concentrations in these animals were 950–120 mg/dl (n=6). Using the same experimental methods as previously described, the contractile responses of aortas from these rabbits were tested by using norepinephrine and serotonin. Relaxation responses to methacholine were determined in serotonin (10⁻⁶M) precontracted aortas to verify the presence of intact endothelium in aortas of cholesterol-fed rabbits.

A third set of four animals (for early group and time period) were anesthetized with sodium pentobarbital, and a left thoracotomy was performed. The left ventricle was isolated and catheterized. The animals then received a lethal injection of pentobarbital followed by a 2-minute perfusion with a Joklik-Modified Eagles minimum essential medium (Gibco, Lawrence, MA) equilibrated with 95% O₂/5% CO₂; the aorta was then fixed by perfusion for 20 minutes with a solution of 1% paraformaldehyde and 1.25% glutaraldehyde. The aorta was then removed en bloc. After excess adventitia was removed, the aorta was opened along the longitudinal length by the ventral approach and further fixed for 6 to 8 hours in a paraformaldehyde/glutaraldehyde fixative. Samples from several areas of the distal thoracic aorta were taken for evaluation. Tissue samples were dehydrated through gradual acetones and then critical-point-dried. Dried tissues were coated with gold-palladium and were examined with a JOEL 35 scanning electron microscope. Adherent leucocytes were quantitated by point-counting of the distal thoracic region from scanning electron photomicrographs. Six photomicrographs were taken at a magnification of 440×. Cell counts were performed without knowledge of the experimental group of age and were expressed as cells/mm².

To standardize for any minor differences in the size of the tissue, contractile responses were expressed as a percentage of the response to 40 mM KCl. The results are expressed as the mean ± SEM. Student’s t test for unpaired observations was used to determine significant differences between groups, and an analysis of variance was used to determine significant differences within groups. In all cases, p<0.05 was considered significant.

Results

Maximal contractile responses to 40 mM KCl were observed with each aortic segment before the addition of any agent and between different agents studied. There was no significant difference in the KCl-induced maximal tension developed in the aortas from NZW and WHHL rabbits at age 1 month (2.9±0.1, 2.8±0.1 g, respectively) or 6 months (3.4±0.2, 3.9±0.1 g, respectively).

Serotonin produced concentration-dependent contractions of isolated aortic segments from NZW and WHHL rabbits in both age groups (Figure 1). Aortas obtained from 1-month-old rabbits were more sensitive and demonstrated greater maximal contractions to serotonin as compared with aortic segments obtained from 6-month-old rabbits of either strain. Compared with aortas...
from 1-month-old NZW rabbits, 1-month-old WHHL rabbit aortas demonstrated a markedly increased maximal response to serotonin (139%±6% vs. 111%±4%, p<0.01). Similar results were observed in aortas obtained from 6-month-old rabbits. WHHL rabbit aortas demonstrated an increased maximal response (62%±2%, vs. 47%±3%, p<0.01) to serotonin.

Norepinephrine also produced a concentration-related contraction of aortas from NZW and WHHL rabbits (Figure 2). In contrast to the results obtained using serotonin, 1-month-old WHHL rabbit aortas exhibited a diminished maximal contractile response to norepinephrine when compared with 1-month-old NZW rabbit aortas (121%±3% vs. 165%±4%, p<0.01); otherwise, contractile responses changed in parallel in the two groups. In 6-month-old rabbits, aortas from WHHL rabbits were less sensitive to norepinephrine than aortas from NZW animals. The concentration-response curve from 6-month-old WHHL aortas shifted approximately 10-fold to the right of the curve for NZW aortas, had a lower maximal contraction (135%±3% vs. 164%±6%, p<0.01), and had an increased concentration required to produce a threshold contraction.

The concentration-response curves for U46619, a thromboxane-mimetic, are shown in Figure 3. The aortas of 1-month-old WHHL rabbits exhibited a slightly greater maximal contractile response to U46619 when compared with aortas from 1-month-old NZW rabbits (161%±10% vs. 143%±7%, NS). As with serotonin, aortas obtained from 6-month-old NZW and WHHL rabbits demonstrated a smaller maximal response to U46619 than aortas from 1-month-old animals. The contractile response to U46619 was similar in aortas from 6-month-old NZW and WHHL rabbits (maximal contractile responses: 111%±2% for WHHL and 111%±3% for NZW, NS).

Histamine also produced vasoconstriction in aortic rings of NZW and WHHL rabbits (Figure 4). In aortas from 1-month-old animals, the responses to histamine did not differ between WHHL and NZW rabbits with respect to the maximal response (139%±5% vs. 144%±6%, NS). However, in 6-month-old animals, a diminished maximal response (129%±4% vs. 157%±7%, p<0.05) was noted to histamine in WHHL rabbits compared with age-matched NZW controls. Thus, the response to histamine did not change with age in NZW rabbit aortas, but decreased in aortas from WHHL rabbits.

At the conclusion of each experiment, the aortic segments were maximally contracted with 10−5M serotonin, and curves of concentration response to methacholine were obtained. Methacholine reduced tension in the aorta in a concentration-related manner (Figure 5). There were significant reductions in the responses to methacholine in
Figure 3. Contractile responses to U46619 in distal aortic rings from New Zealand White (○) and Watanabe heritable hyperlipidemic (□) rabbits at 1 month (upper panel) and 6 months (lower panel) of age. Data are expressed as % of maximal contraction to 40 mM potassium chloride. Each value is the mean±SEM.

The aortas of the WHHL rabbits compared with NZW aortas at 1 month of age (maximal relaxation: 26±% for WHHL and 43±% for NZW, p<0.05). This difference persisted at 6 months of age (maximal relaxation: 35±% for WHHL and 60±% for NZW, p<0.05).

In an additional set of experiments, 1-month-old NZW rabbits were fed a standard diet supplemented with 2% cholesterol for 2 weeks, and the vasoconstrictor responses to serotonin and norepinephrine were determined in isolated aortic segments. Plasma cholesterol concentrations in the cholesterol-fed rabbits were 950±120 mg/dl (n=6). There were no grossly visible atherosclerotic lesions in the aortas from these animals. Maximal responses to KCl in the aortas of cholesterol-fed NZW rabbits (3.7±0.5 g) did not differ from those obtained in the aortas from WHHL rabbits, but were greater than the response of aortas from 1-month-old NZW rabbits. The responses to serotonin were not significantly different in the aortas obtained from cholesterol-fed NZW rabbits when compared with NZW rabbit aortas (106±1% vs. 111±%3) (Figure 6). The maximal responses were significantly less than those of aortas from WHHL rabbits (106±1% vs. 139±%5, p<0.05). In contrast, aortas obtained from hypercholesterolemic NZW rabbits demonstrated a markedly attenuated maximal vasoconstrictor response to norepinephrine when compared with aortas from normal NZW rabbits (103±1% vs. 165±%4, p<0.05) (Figure 7). Furthermore, the maximal contractile response to norepinephrine in aortas from hypercholesterolemic NZW rabbits was significantly less than the responses of aortas from WHHL rabbits (103±1% vs. 121±%3, p<0.05). Methacholine elicited a concentration-related relaxation in aortas from hypercholesterolemic rabbits that was not different from the responses measured in aortas from 1-month-old control rabbits (maximal relaxation: 36±% for hypercholesterolemia and 35±% for control NZW, NS) (Figure 5).

Morphologic studies revealed that with age there were progressive increases in the number of leukocytes attached to the distal thoracic aortas of WHHL rabbits. At 1 month of age, occasional leukocytes (11±2.4/mm²) were found attached to the aortic endothelium of WHHL rabbits (Figure 8). However, by 6 months of age, there were more leukocytes (71.7±49.8/mm²) attached to the aortic endothelium of WHHL rabbits as compared with age-matched NZW rabbits, where no adherent cells were observed (Figure 8). Aortic tissue from 6-month-old NZW rabbits did not differ markedly in appearance from 1-month-old WHHL or NZW rabbits. Small focal irregularities in the endothelium were noted in the aortas of 1-month-old WHHL rabbits that were not seen in aortas of 1-month-old or
Figure 5. Vasodilator responses to methacholine in aortic tissue precontracted with serotonin (10^-6 M). Rings from New Zealand White (NZW) (●) and Watanabe heritable hyperlipidemic (H) rabbits are compared at 1 month (upper panel) and 6 months (middle panel) of age. Rings from hypercholesterolemic (Δ) and control NZW (●) rabbits are compared at 1 month of age (lower panel). Data are expressed as % relaxation. Each value is the mean ± SEM.

Figure 6. Contractile responses to serotonin in aortic rings from Watanabe heritable hyperlipidemic rabbits (○), normal New Zealand White (NZW) rabbits (●), and NZW rabbits ingesting a 2% cholesterol diet (Δ). Animals were 1 month old. Each value is the mean ± SEM.

Figure 7. Contractile responses to norepinephrine in aortic rings from Watanabe heritable hyperlipidemic rabbits (○), normal New Zealand White (NZW) rabbits (●), and NZW rabbits ingesting a 2% cholesterol diet (Δ). Animals were 1 month old. Each value is the mean ± SEM.

6-month-old NZW rabbits. Irregularities in the endothelium were clearly apparent in the 6-month-old WHHL animals. No adherent platelets were observed in 1-month-old NZW rabbits, 1-month-old WHHL rabbits, or 6-month-old NZW rabbits; however, small numbers of adherent platelets were found in aortas from 6-month-old WHHL rabbits.

Discussion

Homozygous WHHL rabbits developed by serial inbreeding exhibit elevated concentrations of serum cholesterol and triglycerides and have been shown to be devoid of functioning LDL receptors in various tissues. The morphologic characteristics of the progression of atherosclerosis in this model have been described previously. At 1 and 2 months of age, there is no gross evidence of atherosclerosis; however, small num-
bers of leukocytes adhering to the intimal surface and microscopic foci of intimal and medial lipid accumulation have been described.\textsuperscript{14,15} By 6 months of age, gross lesions consisting of flat and raised foam cell lesions, some plaques (atheromas), and microscopic medial lipid deposits were evident in the aorta.\textsuperscript{6,10,11} Older, 12-month-old animals have been shown to develop both coronary atherosclerosis and myocardial fibrosis. Platelet aggregation on the intimal surface has also been observed but only in older WHHL animals with lesions of the abdominal aorta. Thus, the WHHL rabbits exhibit biochemical and clinical features similar to those observed in familial hypercholesterolemia.\textsuperscript{12} These similarities underscore the importance of studies in this model.

In the present investigation, we evaluated contractile responses to vasoactive agents in isolated aortic segments obtained from normal rabbits and from rabbits with genetic hypercholesterolemia. These studies demonstrate selective augmentation of the contractile response to serotonin. Potentiated vasoconstrictor responses to serotonin have been reported in atherosclerotic aortas from 24-month-old WHHL rabbits\textsuperscript{16} and from rabbits and monkeys chronically fed a high-cholesterol diet.\textsuperscript{17,18} Enhanced responses were also noted in the denuded endothelialized canine coronary artery.\textsuperscript{19} In contrast to previous studies in the WHHL or cholesterol-fed atherosclerotic rabbits, we have shown that alterations in the sensitivity to serotonin precede any evidence of gross atherosclerosis. Enhanced responses were observed in aortas from 1-month-old WHHL rabbits. In contrast to previous studies, this finding suggests that the alterations in the sensitivity to serotonin are not necessarily a generalized characteristic of atherosclerosis. Our study of diet-induced hypercholesterolemic rabbits further demonstrates that elevations in plasma cholesterol concentrations per se do not produce an increased sensitivity to serotonin. This finding agrees with recent studies of 1-month-old, cholesterol-fed rabbits.\textsuperscript{17} Hypercholesterolemia, however, may be a significant factor in the diminished response to norepinephrine, since the vasoconstrictor response to norepinephrine was reduced in both the WHHL rabbit aortas and the hypercholesterolemic NZW rabbit aortas. Yokoyama and Henry\textsuperscript{a} have previously shown that cholesterol added to the tissue baths augmented the responses of isolated arteries to the vasoconstrictor effects of CaCl\textsubscript{2} and KCl. The present study failed to demonstrate an increased response to KCl in aortic rings from WHHL rabbits compared with NZW rabbits. However, the response to KCl was augmented in vessels from cholesterol-fed NZW rabbits when compared with age-
matched controls. Heistad et al. found that hypercholesterolemia in monkeys augmented the vasoconstrictor responses to norepinephrine, while monkeys with atherosclerosis demonstrated increased responses to only serotonin. The discrepancy between our findings and the findings of Heistad et al. is most likely related to the vascular tissue under investigation. The effect of hypercholesterolemia on the responses to norepinephrine appears to occur at the level of resistance vessels. The present study measured the contractile responses of large capacitance vessels.

A mechanism that may explain our findings relates to alterations in the serotonin receptors of the vascular smooth muscle or vascular endothelium. Nanda and Henry have demonstrated increases in both serotonin and \( \alpha \)-adrenergic receptors in the aortas of cholesterol-fed, atherosclerotic rabbits. We have not measured serotonin receptors in WHHL rabbits. However, to explain the current findings, any alterations in the density of serotonin receptors would have to precede the development of atherosclerosis. This suggests that, coincident with the genetic abnormality in LDL receptors, other receptor abnormalities may occur in these animals. Alternatively, a deficiency in the endothelial production of the vasodilator, prostacyclin, might explain the present findings. However, in a preliminary report, we showed that the synthesis of prostacyclin was augmented in response to either arachidonic acid or serotonin in isolated aortas from 1-month-old WHHL rabbits when compared with aortic segments from 1-month-old NZW rabbits.

Interestingly, the vasodilatory responses to methacholine were less in both 1-month-old and 6-month-old WHHL rabbits when compared with NZW rabbits. Thus, as with serotonin, the reduced responses to methacholine preceded the development of gross atherosclerosis in these animals. No differences in methacholine-induced relaxations were observed between control NZW and hypercholesterolemic NZW rabbits, indicating that, as with the serotonin responses, the condition of hypercholesterolemia alone does not explain the altered responses in aortas of 1-month-old WHHL rabbits. Verbeuren and coworkers reported a reduced vasodilation with acetylcholine in cholesterol-fed rabbits. They attributed this effect to intimal thickening and lipid accumulation producing a barrier that prevented the endothelial vasodilatory factor from reaching the smooth muscle. A possible reason for the differences in our data is the age of the rabbits and the length of cholesterol feeding. Because the rabbits in our present study were fed cholesterol for only 2 weeks, no intimal thickening or medial lipid accumulation occurred. Thus, methacholine-induced relaxations in hypercholesterolemic rabbits were not different from those in control rabbits. In 1-month-old WHHL rabbits exhibiting an impaired vasodilatory response, there also is no intimal thickening or medial lipid accumulation. Thus, these data would suggest an alternative mechanism for the decrease in methacholine vasodilation in WHHL rabbits.

More recently, studies have demonstrated the accumulation of leukocytes on the intimal surface of aortas from WHHL rabbits as early as 1-month of age. These leukocytes have features of monocytes. The number of adherent leukocytes was small in vessels from 1-month-old animals, but they increase progressively at 3, 6, and 12 months. Platelet aggregates on the intimal surface have been observed only in older animals with grossly visible lesions. The role of macrophages in atherosclerotic plaque morphogenesis has been ascribed to their ability to accumulate cholesterol, metabolize cholesterol, and secrete factors that promote smooth muscle cell proliferation. However, macrophages and leukocytes are also capable of synthesizing and releasing prostaglandins, thromboxanes, and leukotrienes. These agents may act directly to cause vasoconstriction or indirectly to augment the responses to other vasoconstrictor agents. Thus, our findings, in conjunction with the morphologic studies of others, suggest that altered responses to vasoconstrictor agents occur at an age when the only observed pathologic abnormality is the early stage of leukocyte accumulation on the aortic wall. Although we have not systematically correlated the presence of these cells with altered vasoconstrictor responses, our findings suggest a potential role for monocyte-derived macrophages and possibly other leukocytes in modulating the vasoconstrictor responses to certain vasoactive agents.

In summary, these studies demonstrate a specific and selective increase in the vasoconstrictor response of isolated aorta to serotonin in a genetic model of hypercholesterolemia. These findings suggest that enhanced vasoconstriction may occur in response to serotonin during the earliest stages of atherosclerotic plaque morphogenesis.

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