Interaction between mild hypercholesterolemia, HDL-cholesterol levels, and angiotensin II in intimal hyperplasia in mice

Valdeci da Cunha,1,* Baby Martin-McNulty,* Jon Vincelette,* Lening Zhang,† John C. Rutledge,† Dennis W. Wilson,§ Ronald Vergona,* Mark E. Sullivan,* and Yi-Xin Wang§

Department of Pharmacology,* Berlex Bioscience, Richmond, CA; and Department of Internal Medicine,† School of Medicine, and Department of Pathology, Microbiology, and Immunology,§ School of Veterinary Medicine, University of California at Davis, Davis, CA

Abstract Two month old C57BL/6 mice were placed on three different diets: 1) normal diet (NC; 0.025% cholesterol), 2) hypercholesterolemic Western-type diet (HC-W; 0.2% cholesterol), and 3) hypercholesterolemic Paigen-type diet (HC-P; 1.25% cholesterol plus 0.5% cholic acid). At 6 months of age, the animals underwent ligation of the left carotid artery and were randomly assigned to vehicle (PBS, subcutaneous) or angiotensin II (Ang II; 1.4 mg/kg/day, subcutaneous) treatment for 4 weeks. Low density lipoprotein-cholesterol levels were similarly increased in both HC diets (NC, 4 ± 3 mg/dl; HC-W, 123 ± 17 mg/dl; HC-P, 160 ± 14 mg/dl). However, the levels of high density lipoprotein-cholesterol (HDL-C) were reduced only in animals fed the HC-P diet (NC, 82 ± 6 mg/dl; HC-W, 79 ± 7 mg/dl; HC-P, 58 ± 7 mg/dl). In Ang II-treated mice, carotid artery ligation induced intimal smooth muscle cell proliferation to a similar extent in NC- and HP-W-fed animals. However, a significantly larger intimal area developed in ligated vessels from Ang II-treated mice fed the HC-P diet (3.6-fold higher than in Ang II-treated NC mice). Together, these results show the accelerating effect of mild hypercholesterolemia on intimal SMC proliferation, locally produced neurohumoral mediators, particularly angiotensin II (Ang II) (9), may be equally important in determining such predisposition. Increased vascular levels of Ang II and the expression of its AT1 receptors were found in intimal SMCs of aortas from aging nonhuman primates (10) and in in-stent restenotic lesions of human coronary arteries (11). More importantly, in vitro studies showing synergism between lipoproteins and Ang II in inducing decreased cytosolic calcium concentration (12) and DNA synthesis (13) in vascular SMCs has led to the hypothesis that the effect of mild increases of cholesterol levels on SMC proliferation would be amplified by Ang II. Nonetheless, there are no previously reported in vivo data supporting this concept. In this study, we assessed the interaction between mild hypercholesterolemia and Ang II in SMC proliferation in aortas from hypercholesterolemic rabbits. In both studies, circulating levels of cholesterol were 9- and 18-fold higher than control levels, exceeding the pathophysiologic range of human hypercholesterolemia. Additionally, the concomitant development of spontaneous atheromatous plaques found in these studies adds another important level of interaction between macrophage/foam cell-derived mediators and SMC proliferation (7).

In addition to changes in hemodynamic forces (e.g., low shear stress) (8) as predisposing factors for intimal hyperplasia development, locally produced neurohumoral mediators, particularly angiotensin II (Ang II) (9), may be equally important in determining such predisposition. Increased vascular levels of Ang II and the expression of its AT1 receptors were found in intimal SMCs of aortas from aging nonhuman primates (10) and in in-stent restenotic lesions of human coronary arteries (11). More importantly, in vitro studies showing synergism between lipoproteins and Ang II in inducing decreased cytosolic calcium concentration (12) and DNA synthesis (13) in vascular SMCs has led to the hypothesis that the effect of mild increases of cholesterol levels on SMC proliferation would be amplified by Ang II. Nonetheless, there are no previously reported in vivo data supporting this concept. In this study, we assessed the interaction between mild hypercholesterolemia and Ang II in SMC proliferation in aortas from hypercholesterolemic rabbits. In both studies, circulating levels of cholesterol were 9- and 18-fold higher than control levels, exceeding the pathophysiologic range of human hypercholesterolemia. Additionally, the concomitant development of spontaneous atheromatous plaques found in these studies adds another important level of interaction between macrophage/foam cell-derived mediators and SMC proliferation (7).

Supplementary key words high density lipoprotein • smooth muscle cells • vascular remodeling

Proliferation of smooth muscle cell (SMCs) and migration into the intima is the hallmark of restenosis after vascular interventions (angioplasty, stent or vein graft placement) (1–3) and an important feature of preatherosclerotic lesions (4). It is well established that hypercholesterolemia accelerates atherosclerosis development; however, studies addressing the effect of circulating cholesterol on intimal SMC proliferation have yielded conflicting results. For instance, Theilmeier et al. (5) showed that hypercholesterolemia decreased the population of SMCs in atheromatous lesions in postangioplasty porcine coronary arteries. In contrast, in a recent study, Kahn et al. (6) observed accelerated angioplasty-induced intimal hyperplasia in aortas from hypercholesterolemic rabbits. In both studies, circulating levels of cholesterol were 9- and 18-fold higher than control levels, exceeding the pathophysiologic range of human hypercholesterolemia. Additionally, the concomitant development of spontaneous atheromatous plaques found in these studies adds another important level of interaction between macrophage/foam cell-derived mediators and SMC proliferation (7).
METHODS

Animals and experimental design

Two month old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were placed on three different cholesterol diets as follows: 1) normal diet (NC; 6% fat, 0.025% cholesterol), 2) hypercholesterolemic Western-type diet (HC-W; 20% fat, 0.2% cholesterol), and 3) hypercholesterolemic Paigen-type diet (HC-P; 15% fat, 1.25% cholesterol, plus 0.5% cholic acid). All diets were matched for kilocalories, and the animals were allowed free access to food and water. At 6 months of age, the animals underwent ligation of the common left carotid artery with a 5-0 silk suture and were randomly assigned to receive either vehicle (PBS, subcutaneous) or Ang II (1.4 mg/kg/day, subcutaneous) via an osmotic infusion pump (model 2004; Alzet). The right carotid artery was used as a self-control, by placing the suture around it without ligating. After surgery, the animals were returned to their original assigned diet, yielding six experimental groups: NC, HC-W, and HC-P treated with either vehicle or Ang II. Four weeks later, the animals were euthanized, followed by blood withdrawal and dissection of carotid arteries.

Morphometric analysis and immunohistochemistry

The carotid arteries were removed en bloc, fixed in 10% buffered formalin, and then embedded in paraffin. Sections (5 μm) from the middle portion (~3 mm away from the ligation) of both vessels stained with hematoxylin and eosin (H&E) were used for morphometry and morphology evaluation, according to a previous study (14). Contours of the lumen, internal elastic lamina (IEL), external elastic lamina (EEL), and the adventitial layer when present (a tightly packed agglomerate of cells surrounding the EEL) were traced on digitized images using a computer-based morphometric analyzing system (Computer-Assisted Stereology Toolbar; Olympus Denmark).

Vessel and lumen diameters, defined as EEL perimeter/π and lumen perimeter/π, respectively, were calculated assuming that all vessels have a circular morphology. Adventitial area represented the area between the EEL and the outer edge of tightly packed adventitial cells. Medial area represented the area between the EEL and the IEL, and intimal lesion area was calculated by subtracting lumen area from the IEL area. Similar to area, the thickness of adventitial, medial, and intimal layers was calculated by dividing the subtracted perimeter by 2 (e.g., advent-
itial thickness = adventitial layer perimeter / EEL perimeter/2). Lumen radius was obtained by dividing lumen diameter by 2.

For smooth muscle α-actin immunohistochemical analysis, sections were stained with a DAKO monoclonal anti-α-actin (clone 1A4, 1:100 dilution). Macrophage MAC-3 immunostaining was performed with an antibody (1:125 dilution; BD Pharmingen) previously shown to recognize its target epitope in formalin-fixed, paraffin-embedded tissues (15). Positive and negative controls were run for both antibodies according to the manufacturer’s recommendations.

Cholesterol profile determination

Total cholesterol and lipoprotein cholesterol fractions were measured in fasting serum by a standard clinical laboratory (IDEXX, Sacramento, CA) using commercially available kits as follows: total cholesterol with the Cholesterol Assay kit (Equal Diagnostics), and high density lipoprotein-cholesterol (HDL-C) with the HDL Direct Liquid Select kit (Equal Diagnostics). Low density lipoprotein-cholesterol (LDL-C) was calculated using the equation LDL-C = total cholesterol − HDL-C − (triglycerides/5).

Statistical analysis

The results are presented as averages ± SEM. Statistical analysis was performed by one-way ANOVA followed by the Newman-Keuls test. P < 0.05 was considered statistically significant.

RESULTS

Lipid profile

As shown in Fig. 1, the long-term feeding of different dietary cholesterol content resulted in different circulating cholesterol profiles at the end of experiment. The two high-cholesterol diets induced an ~2.5-fold increase in total circulating cholesterol, reflecting increased LDL-C levels, which were virtually absent in mice fed the NC. LDL-C levels were similarly increased in HCP and HCW groups. However, HDL-C levels were significantly reduced only on the HCP diet, resulting in a significantly higher LDL-C/HDL-C ratio in this group compared with animals fed the HC-W diet. Ang II treatment did not significantly affect lipid profile, including the LDL-C/HDL-C ratio, in any of the three dietary regimens, except for higher levels of total cholesterol and HDL-C in the HCW group.

Carotid artery morphology

As shown in Fig. 2, we first assessed the changes in the thickness of the vessel wall layers and lumen radius after carotid artery ligation, in relation to the nonligated (control) artery. In agreement with previous observations (16), there was no significant change in the vessel wall in vehicle-treated NC mice. Although carotid artery ligation

![Fig. 2. Vascular wall layer thickness and lumen radius of ligated and nonligated (control) carotid arteries from mice fed three different cholesterol diets treated with vehicle or Ang II. Results represent averages ± SEM. * P < 0.05 versus vehicle within the same diet group; ** P < 0.05 versus the corresponding segment in the control artery.](https://via.placeholder.com/150)
induced lumen reduction in vehicle-treated mice fed the two hypercholesterolemic diets, a significant intimal thickening was observed only in mice fed the HC-P diet. Mice fed the HC-P diet also displayed adventitial proliferation in both ligated and control arteries. In Ang II-treated mice, however, the ligation induced intimal hyperplasia, adventitial proliferation, and lumen reduction in all three diets. An additional effect of Ang II was a significant expansion of lumen in control arteries from NC mice, compared with control arteries in the vehicle-treated group.

We next analyzed the differences among the six groups regarding intimal, medial, and adventitial areas of ligated vessels only (Fig. 3). None of the animals on the NC diet developed intimal hyperplasia after carotid artery ligation. Although not significantly different from the NC group, one in seven mice fed the HC-W diet and three in six fed the HC-P diet developed intimal hyperplasia. In Ang II-treated mice, carotid artery ligation induced intimal SMC proliferation to a similar extent in NC- and HP-W-fed animals. However, a significantly larger intimal area developed in ligated vessels from Ang II-treated mice fed the HC-P diet (3.6-fold higher than in Ang II-treated NC mice). Carotid artery ligation induced a significant adventitial proliferation only in mice fed the HC-P diet but not in those fed the NC or HC-W diet. Ang II-treated mice developed a thicker neoadventitial layer after carotid artery ligation to a similar extent regardless of the diet.

To evaluate the relationship between intimal hyperplasia and vascular remodeling, vascular and luminal diameters of ligated arteries are shown in Fig. 4. Ligated vessels from the HC-P group underwent significant expansion compared with those from the NC and HC-W groups. Vascular expansion was also observed in Ang II-treated mice in all three diet groups compared with their respective vehicle-treated groups. A further expansion of the vessel was seen in Ang II-treated animals fed the HC-P diet, which was also significantly different from the Ang II treatment on both NC and HC-P diets. The development of intimal hyperplasia in the vehicle-treated HC-P group was not accompanied by a significant reduction in lumen diameter. Although not statistically significant, average lumen diameter was slightly larger in all Ang II-

![Graph](image1)

**Fig. 3.** Intimal, medial, and adventitial areas of ligated carotid arteries from mice fed three different cholesterol diets treated with vehicle or Ang II. Results represent averages ± SEM. *P < 0.05 versus vehicle within the same diet group; **P < 0.05 versus the same treatment in the NC diet group; †P < 0.05 versus the same treatment in the HC-W diet group.

![Graph](image2)

**Fig. 4.** Vessel and lumen diameters of ligated carotid arteries from mice fed three different cholesterol diets treated with vehicle or Ang II. Results represent averages ± SEM. *P < 0.05 versus vehicle within the same diet group; **P < 0.05 versus the same treatment in the NC diet group; †P < 0.05 versus the same treatment in the HC-W diet group.
treated mice compared with their respective vehicle-treated groups.

H&E- and SMC α-actin-stained sections of representative ligated arteries are shown in Fig. 5. Intimal lesions were characterized by proliferating smooth muscle cells (α-actin-positive) in a matrix background. The arteries from Ang II-treated mice in all three diets and those from vehicle-treated HC-P mice underwent significant enlargement and displayed thicker neoadventitial proliferating areas. Bar = 200 and 50 μm for H&E and α-actin, respectively.

Fig. 5. Representative sections of ligated arteries stained with hematoxylin and eosin (H&E) and α-actin from mice fed three cholesterol diets treated with vehicle (upper rows) or Ang II (lower rows). Intimal lesions were characterized by proliferating smooth muscle cells (α-actin-positive cells) in a matrix background. The arteries from Ang II-treated mice in all three diets and those from vehicle-treated HC-P mice underwent significant enlargement and displayed thicker neoadventitial proliferating areas. Bar = 200 and 50 μm for H&E and α-actin, respectively.

DISCUSSION

Intimal SMC proliferation is an important feature of stenotic (17) and preatherosclerotic (4) lesions and the hallmark of restenosis after vascular interventions (angioplasty, stent and graft placement) (1–3). Therefore, understanding the regulatory process of intimal SMC proliferation is of crucial importance to the optimization of therapies targeting arterial stenosis/restenosis. Although hypercholesterolemia is indubitably a major risk factor for atherosclerotic plaque formation, it is unclear how it affects intimal SMC proliferation. Theilmeier et al. (5) described a sparse SMC population in postangioplasty atherosclerotic lesions in porcine coronary arteries, suggesting a negative effect of hypercholesterolemia on SMC proliferation. In a similar study, Kahn et al. (6) observed a positive correlation between postangioplasty lesion size and blood cholesterol levels in the aorta of hypercholesterolemic rabbits. In the latter study, however, the relative proportions of SMCs and other cellular components of the lesion were not determined. Plasma cholesterol levels achieved in these studies, which far exceeded the pathophysiologic range of human dyslipidemia, were associated with the development of complex intimal atherosclerotic lesions. Under such conditions, it would be virtually impossible to directly assess the role of hypercholesterolemia on SMC proliferation without the interfering effect that macrophage/foam cell-derived mediators would have on these cells. Indeed, Bacakova, Herget, and Wilhelm (7) observed that the macrophage-
induced degradation of matrix is associated with reduced adhesion and migration of SMCs. In this study, we induced mild hypercholesterolemia in C57BL/6 mice, taking into consideration previous reports on the relative resistance of this strain to developing exaggerated hypercholesterolemia (18, 19).

Reduction of endothelial shear stress is considered to be the primary mechanism underlying intimal hyperplasia in the carotid artery ligation model (14), simulating the low shear-induced intimal growth in atherosclerosis-prone sites of human coronary arteries (20) and in arterial segments submitted to angioplasty (8). Other important features of this model are the absence of intraluminal thrombus and inflammatory infiltrate, potent stimuli for SMC proliferation (21, 22). Because we aimed to assess the accelerating effect of hypercholesterolemia and Ang II on lesion development, we chose to analyze the middle portion of the vessel, away from the immediate vicinity of the ligature, where occluding lesions develop (14, 23). In this study, the vessel is still subject to arterial blood pressure and pulsation (16), resembling human vascular segments exposed to low shear stress. Our results showing no lesions at the middle portion of the vessel in vehicle-treated normocholesterolemic mice are consistent with previous observations (16). Therefore, like human vascular disease, in our approach the vessel ligation creates an environment in which different risk factors can be evaluated.

In this study, the two hypercholesterolemic diets induced similar increases in total cholesterol, despite significant differences in cholesterol content (0.2% in the HC-W diet vs. 1.25% in the HCP diet). This finding, however, is in agreement with previous studies in which the increase of total cholesterol in C57BL/6 mice fed the HC-P diet (2.1- to 2.9-fold) (24–26) was comparable with that in animals fed the HC-W diet (2.2-fold) (27). In contrast with a widely accepted concept, addition of fat to the hypercholesterolemic diet, not cholate, increases cholesterol absorption (28). Although total fat accounts for 21% of the composition of the HC-W diet used in this study, it represents only 15% in the HC-P diet. Interestingly, in this study, HDL-C levels were significantly reduced only in the HC-P group. This result is consistent with previous reports showing that mice fed cholate-containing diets present reduced circulating levels of HDL-C (24, 29). Although the precise mechanism for this effect is unknown, a reduction of HDL synthesis and increased dependence on HDL-derived cholesterol for bile acid synthesis have been proposed (30). Additionally, the ability of HDL to remove cholesterol from peripheral tissue and return it to the liver might be also compromised in mice fed the HC-P diet (31). Although mouse models meet several criteria regarding hypercholesterolemia in human patients (32), mice carry most of their plasma cholesterol on HDL, which clearly does not represent the human cholesterol profile. However, a role in reverse cholesterol transport and antiinflammatory properties, important mechanisms by which HDL exerts its antiatherogenic action in humans, have also been demonstrated for the murine lipoprotein (33, 34).

In this study, Ang II promoted the formation of intimal hyperplasia in ligated carotid arteries. This effect is not unexpected, because Ang II is able to activate a variety of intracellular pathways involved in SMC migration and proliferation (35–37). It should be noted that the proliferative effect seen here was limited to hemodynamically altered vessels. These results suggest a greater impact of the mitogenic action of Ang II on arterial vessel segments exposed to low shear stress. In support of this, in a separate study, we observed that the production of monocyte chemoattractant protein-1, a recognized inducer of SMC proliferation (38), was significantly higher in ligated arteries from Ang II-treated C57BL/6 mice compared with contralateral nonligated arteries (data not shown).

Another important finding of this study was that Ang II-induced intimal hyperplasia in ligated arteries was significantly increased in dyslipidemic (increased LDL-C and reduced HDL-C) mice, suggesting an additive effect between the two risk factors. This finding confirms in vitro studies showing that the increase of cytosolic calcium concentration and phosphoinositide content by LDL-C in SMCs was enhanced by Ang II (12). Such an interaction was also shown for the induction of SMC proliferation (13). Although the results of these studies suggest that hypercholesterolemia might play a role in regulating the proliferative response of SMCs to Ang II, our study is the first in vivo report to provide direct evidence for this hypothesis.

Although largely ignored in the past, adventitial and perivascular reactions have gained substantial attention regarding their role in vascular remodeling after vascular interventions (39). However, as suggested by our results, the mechanisms that induce adventitial proliferation might be different from those involved in intimal hyperplasia. Although intimal hyperplasia developed only in the ligated arteries, neoadventitia was also present in normal arteries of mice fed the HC-P diet and in those treated with Ang II. It should be mentioned that proliferation of adventitial fibroblast is stimulated by oxidative stress (40), a process that has been shown to be exacerbated by both hypercholesterolemia (41) and Ang II (42) in the vasculature of C57BL/6 mice.

Constrictive vascular remodeling, characterized by internal elastic layer recoil, also accounts for postangioplasty restenosis (43). In this study, expansion of the EEL (and IEL) was observed in all treatments that induced intimal hyperplasia, explaining the relative maintenance of lumen diameter. Although we do not have a definite explanation for this result, we hypothesize that the participation of matrix metalloproteinases may also play a role. In agreement, Mason et al. (44) have shown that the overexpression of matrix metalloproteinase-9 in an injured rat carotid artery led to an increase in SMC migration and expansive remodeling. Additionally, our data suggest that both stimuli may not be involved in the internal elastic layer recoil accompanying angioplasty. Regardless of the mechanism, our data indicate similarities between the combination of mild hypercholesterolemia-reduced cholesterol levels and angiotensin II in intimal hyperplasia 481
HDL-C levels and Ang II actions on expansive vascular remodeling associated with intimal hyperplasia. In conclusion, we have shown that the combination of mild hypercholesterolemia, reduced HDL-C levels, and Ang II accelerates intimal hyperplasia in hemodynamically altered vessels.

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