Vascular Function During Prolonged Progression and Regression of Atherosclerosis in Mice

Jordan D. Miller, Yi Chu, Lauren E. Castaneda, Kristine M. Serrano, Robert M. Brooks, Donald D. Heistad

Objective—Endothelial dysfunction is associated with atherosclerosis in mice, but it is difficult to reduce cholesterol levels enough to study regression of atherosclerosis in genetically modified mice. The goal of this study was to examine vascular structure and function before and after reducing elevated plasma lipid levels with a genetic switch in Reversa mice, and identify novel mechanisms contributing to structural and functional improvements in the vasculature after reduction of blood lipids.

Methods and Results—After 6 months of hypercholesterolemia, endothelial function (maximum relaxation to acetylcholine) in aorta was impaired and responses to nitric oxide were unaffected. Further impairment in endothelial function was observed after 12 months of hypercholesterolemia and was associated with reductions in sensitivity to nitric oxide. Expression of dihydrofolate reductase was reduced at 6 and 12 months, and addition of the tetrahydrobiopterin precursor sepiapterin significantly improved endothelial function. Reducing cholesterol levels at 6 months normalized dihydrofolate reductase expression and prevented further impairment in endothelial function. Similar functional changes were observed after 12 months of hypercholesterolemia followed by 2 months of lipid lowering.

Conclusion—Our data suggest that endothelial dysfunction after prolonged hypercholesterolemia is the result of both impairment of sensitivity to nitric oxide and reduced nitric oxide synthase cofactor bioavailability. Both of these changes can be prevented by normalizing blood lipids during moderately severe or advanced atherosclerosis.

Key Words: atherosclerosis ■ endothelial function ■ lipids ■ vascular biology

Endothelial dysfunction in humans with atherosclerosis is a result of an increase in oxidative stress, reduction in nitric oxide bioavailability, and depletion of essential nitric oxide synthase (NOS) cofactors.1–5 Endothelial dysfunction is also evident in early stages of atherosclerosis in hypercholesterolemic mice. Pharmacological and genetic interventions indicate that both oxidative stress and nitric oxide bioavailability are important determinants of plaque initiation, progression, and stability.6–11 It has been difficult, however, to study structural and functional consequences of regression of atherosclerosis in low-density lipoprotein receptor-null (LDLr–/–) and apolipoprotein E–null (Apoe–/–) mice. Dietary restriction, for example, does not adequately lower cholesterol levels to study plaque regression, and lipid-lowering drugs are relatively ineffective or have significant off-target effects.12,13

In Reversa mice, when cholesterol levels are reduced through conditional deletion of the microsomal triglyceride transfer protein (mttp) at an early age, initiation of atherosclerotic plaque formation is prevented.14 When moderately advanced plaques are present, reduction of blood lipids results in rapid macrophage emigration, reduced inflammation, decreased lipid content, and increased collagen content in atherosclerotic plaques.15 Functional consequences of these changes, however, are not known.

In the present study, we used Reversa mice to examine effects of prolonged progression and regression of atherosclerosis on vascular function and gene expression. Our primary goal was to test the hypothesis that lipid-lowering with a genetic switch would normalize endothelial function after prolonged progression of atherosclerosis. Our second goal was to identify mechanisms underlying changes in endothelial function after reduction of blood lipids, and to determine whether there is a strong relationship between improvements in endothelial function and reductions in expression of NAD(P)H oxidase.

Materials and Methods

Animals

We studied female LDLr–/– mice that were homozygous for apolipoprotein B100–only allele, a conditional knockout allele of Mttp, and an Mx1-Cre transgene (Ldlr–/–Apob<super>100/100</super>Mttp<super>fl/fl</super>Mx1Cre<sup>+/-</sup>, a
Histological and Immunohistochemical Changes in Aorta

Serial sections (10 μm thickness) were taken from tissue blocks frozen in Optimal Cutting Temperature Compound (OCT; Sakura Finetek). Lipid deposition was measured using Oil Red O (Sigma, France).\(^{3,6,17}\) Calcification was measured using Alizarin Red staining (Sigma, France).\(^{3,6,17}\) Macrophages were identified using F4/80 staining (AbD Serotec).\(^{16}\) Images were obtained using light microscopy at ×4 and ×10 magnification (Olympus BX 51 Digital Light Microscope, Olympus, Japan). For analysis, we used Adobe Photoshop CS2 (version 7, Adobe Systems Inc, San Jose, CA) to select only pixels that express red staining, as described previously.\(^{3,6,17}\) Data are expressed as the percentage of vessel area that displays positive staining.

Gene Expression

Tissue from the aortic arch and proximal descending thoracic aorta was used to measure gene expression. Quantitative real-time PCR was used to measure expression of genes related to calcification (Mmx2, core binding factor α1, osteonectin, and osteopontin), lipid deposition, and reverse cholesterol transport (ATP-binding cassette subfamily A1 and B1 [ABCA1 and ABCG1]), antioxidant defense mechanisms (manganese superoxide dismutase [SOD], copper-zinc SOD, and extracellular SOD), prooxidant mechanisms (NADPH oxidase catalytic subunits 1, 2, and 4 [Nox1, Nox2, and Nox4], respectively), and enzymes related to synthesis of nitric oxide (NOS isoforms 1, 2, and 3 [NOS1, NOS2, and NOS3], GTP cyclohydrolase-1 [GTPCH], and dihydrololate reductase [DHFRR]), using previously described methods.\(^{3}\)

Vasomotor Function

Vasomotor function of aorta was evaluated by measurement of isometric tension ex vivo, as we have described previously.\(^{3}\) Briefly, mice were anesthetized and euthanized with an overdose of sevoflurane. The aorta was excised, loose connective and adipose tissue were removed, and the aorta was placed in oxygenated Krebs buffer. Vessels were suspended between 2 triangular hooks in an organ bath, and isometric tension was measured. Vessels were preconstricted to 50% to 60% of maximum tension with prostaglandin-F\(_2\alpha\), and responses to acetylcholine (endothelium-dependent), and papaverine (nitric oxide-independent) were examined.

We used the SOD mimetic Tempo (1 mmol/L dissolved in saline; Sigma) and sepiapterin (100 μmol/L, dissolved in saline; Sigma). Adjacent regions of descending thoracic aorta were used as time controls for antioxidant/sepiapterin treatment conditions, and incubated in the organ bath with identical concentrations of vehicle. Because we did not detect a significant time effect across any of the conditions, data are presented as the mean of all time control vessels.

Statistical Analyses

All data are expressed as mean±SE. Differences in contraction and relaxation across groups were detected using an analysis of variance, with subsequent post hoc testing using Bonferroni-corrected t tests.

Results

Changes in Blood Lipids

In control mice, plasma cholesterol levels were 157±20 at 6 months and 145±27 mg/dL at 12 months. In hypercholesterolemic mice, plasma cholesterol levels were 997±87 at 6 months and 828±89 mg/dL at 12 months (P<0.05 versus control mice at both time points). In the regression group, switching off the mttp gene reduced plasma cholesterol levels to 228±30 mg/dL (P<0.05 versus 6 month or 12 month hypercholesterolemic mice).

Histological and Immunohistochemical Changes in Aorta

In control mice, there was negligible lipid deposition and intimal plaque formation at 6 and 12 months. In contrast, lipid deposition and intimal plaque formation were significantly increased in aorta from hypercholesterolemic mice at 6 months, and continued to increase at 12 months (Figure 1A). In the regression group, normalizing blood lipids after 6 months of hypercholesterolemia prevented increases in lipid content at 12 months (P<0.05 versus 12-month hypercholesterolemic mice), and tended to reduce (P=n.s.) intimal plaque size at 12 months (Figure 1 in the online-only Data Supplement). Changes in macrophage infiltration paralleled changes in lipid content of intimal plaques (Figure II in the online-only Data Supplement).

Vascular calcium deposition was negligible in control mice at 6 and 12 months (Figure 1B), but increased progressively from 6 to 12 months in hypercholesterolemic mice (expressed as absolute calcium levels [872±173 and 1870±324 pixels, respectively] and percentage of vessel positively stained; Figure 1B). Normalizing lipid levels after 6 months of hypercholesterolemia markedly attenuated increases in calcium in aorta from 6 to 12 months (absolute calcium levels=360±160 pixels; percentage of vessel positively stained; Figure 1B).

Gene Expression in Aorta

Expression of ABCA1 was significantly elevated after 6 and 12 months of hypercholesterolemia compared with control mice (Figure 1C). Reducing cholesterol levels after 6 months of hypercholesterolemia significantly reduced ABCA1 expression at 12 months (Figure 1C). ABCG1 expression was significantly elevated after 6 months of hypercholesterolemia, but did not remain elevated after 12 months of hypercholesterolemia or after normalizing cholesterol levels at 6 months (Figure 1E).

Expression of runt-related transcription factor 2 was not significantly affected by any of the interventions used in this study (Figure 1D). Osteopontin, however, was significantly increased after 6 or 12 months of hypercholesterolemia relative to normocholesterolemic control mice (Figure 1F). Normalizing cholesterol levels after 6 months of hypercholesterolemia significantly reduced expression of osteopontin at 12 months (Figure 1F).

Changes in Vasomotor Function

Endothelial function and responses to SNP were normal in 6- and 12-month-old control mice (maximum relaxation to acetylcholine and SNP were 75% and 90%, respectively; see Figure 2A–2D). After 6 months of hypercholesterolemia, endothelial function was significantly impaired (Figure 2A)
with no detectable impairment in vascular relaxation to SNP (see Figure 2C). After 12 months of hypercholesterolemia, endothelial function was profoundly impaired (Figure 2B) and was also associated with impaired relaxation to SNP (Figure 2D). Normalizing cholesterol levels after 6 months of hypercholesterolemia prevented impairment in vascular responses to acetylcholine and SNP observed in hypercholesterolemic mice at 12 months. Responses to prostaglandin F2α were impaired only in 12-month hypercholesterolemic mice (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 6 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F). Similar changes were observed in other mice in which cholesterol was normalized for 2 months after 12 months of hypercholesterolemia (Figure 2E and 2F).

Expression of NOX Isoforms and Genes Related to Redox Balance
Expression of endothelial nitric oxide synthase was not affected by any interventions in this study (Figure 3C). Expression of inducible nitric oxide synthase was significantly increased after 6 or 12 months of hypercholesterolemia. Normalizing cholesterol levels after 6 months of hypercholesterolemia significantly reduced inducible nitric oxide synthase expression at the 12-month time point (Figure 3B). Expression of neuronal nitric oxide synthase was significantly increased after 12 months of hypercholesterolemia (Figure 3A), but was not altered by normalizing cholesterol levels for 6 months.

Expression of copper–zinc SOD, manganese SOD, and extracellular SOD was significantly reduced in 6- and 12-month hypercholesterolemic mice compared with isotime normocholesterolemic mice (Figure 3D–3F). Normalizing cholesterol levels after 6 months of hypercholesterolemia significantly increased expression of copper–zinc SOD and extracellular SOD, but not manganese SOD, at 12 months (Figure 3D–3F).

Expression of NOX1 was not affected by any interventions in this study (Figure 3G). Expression of NOX2 was significantly increased in hypercholesterolemic mice at 6 and 12 months, when compared with control mice (Figure 3H). In contrast, expression of NOX4 was significantly reduced after 6 or 12 months of hypercholesterolemia (Figure 3I). Reducing cholesterol levels for 6 months did not affect NOX2 and NOX4 expression compared with 12-month hypercholesterolemic mice (Figure 3H and 3I).

Effects of Exogenous Antioxidants on Vascular Function
Addition of Tempo to the organ chamber baths did not improve vascular function in 6- or 12-month control animals (Figure 4A and 4B). Furthermore, Tempo tended to impair vascular function in some groups of mice at 6 and 12 months (Figure 4A and 4B), but these changes did not reach statistical significance. Responses to SNP were not significantly affected by Tempo.

Expression of Genes Related to Tetrahydrobiopterin Production
Expression of GTPCH was significantly increased after 6 and 12 months of hypercholesterolemia, when compared with control animals (Figure 5A). Normalizing cholesterol levels after 6 months of hypercholesterolemia reduced expression of GTPCH compared with 12-month hypercholesterolemic mice (Figure 5A). In contrast, expression of DHFR was significantly reduced after 6 and 12 months of hypercholesterolemia (Figure 5A). Normalizing cholesterol levels for 6 months normalized DHFR expression at 12 months (Figure 5B).

Effects of Exogenous Tetrahydrobiopterin Precursors on Vascular Function
Addition of sepiapterin to the organ chamber baths did not improve vascular function in 6- or 12-month control animals (Figure 5A). Normalizing cholesterol levels after 6 months of hypercholesterolemia reduced expression of GTPCH compared with 12-month hypercholesterolemic mice (Figure 5A). In contrast, expression of DHFR was significantly reduced after 6 and 12 months of hypercholesterolemia (Figure 5A). Normalizing cholesterol levels for 6 months normalized DHFR expression at 12 months (Figure 5B).

Discussion
The goals of this study were to examine effects of reducing cholesterol levels on structure and function of the aorta after...
prolonged hypercholesterolemia in mice and examine mechanisms contributing to these changes. We observed in mice, as previously reported in other species, that reducing cholesterol levels prevents atherosclerotic plaque lipid content and prevents further endothelial dysfunction and loss in vascular nitric oxide sensitivity resulting from prolonged hyperlipidemia. The major novel findings of this study are as follows: (1) dystrophic calcium accumulation in aorta is remarkably responsive to reductions in blood lipids in mice; (2) expression of antioxidant enzymes, but not prooxidant enzymes, is favorably affected by reducing blood lipids in mice; and (3) restoration of tetrahydrobiopterin synthesis and salvage pathways may be an important mechanism contributing to improvement in endothelial function after reduction in blood lipids.

**Histological Changes During Progression and Regression of Atherosclerosis in Mice**

In the present study, hypercholesterolemia resulted in progressive increases in lipid in the aorta. ABCA1 and ABCG1, which are genes critical for reverse cholesterol transport and prevention of lipid accumulation in atherosclerosis, were significantly elevated after 6 months of atherosclerosis. After 12 months of hypercholesterolemia, ABCA1 (but not ABCG1) was elevated over control levels. Although global reduction of ABCA1 accelerates progression of atherosclerosis in hypercholesterolemic mice, overexpression attenuates atherosclerotic lesion size only when overexpressed in endothelium. Conversely, global reduction of ABCG1 slows progression of atherosclerosis but may exert atheroprotective effects if increased only in the vascular endothelium. Collectively, these data suggest that increases in endothelial ABCA1 and ABCG1 may be protective in early stages of atherogenesis, with progressive reductions in ABCG1 contributing to lipid accumulation in advanced stages of vascular disease.

We also observed calcium deposition in aorta with prolonged hypercholesterolemia, which was surprisingly responsive to lipid-lowering. Although increases in calcium were not associated with increases in Runx2, Msx2, or osteix, osteopontin expression paralleled changes in vascular calcium levels. This pattern of gene expression is consistent with observations from dystrophically calcified tissue, where ectopic calcium accumulation progresses in the absence of increased expression of markers of osteoblast-like cells or evidence of bone matrix formation. These data suggest that even dystrophic calcification may be capable of regression after reductions in blood lipids in mice. These data differ from previous studies in humans and nonhuman primates, where calcification rarely undergoes resorption after reduction of blood lipids. Understanding the differences between species and therapeutic interventions (eg, dietary intervention versus statin treatment versus mttp inactivation) may be critical to application of these findings to therapeutic interventions in humans.

**Vasomotor Function and Redox-Related Genes During Progression and Regression of Atherosclerosis in Mice**

**Endothelium-Dependent Relaxation**

Relaxation to acetylcholine was significantly impaired after 6 and 12 months of hypercholesterolemia. Altered responses are likely to be the result of increases in oxidative stress resulting from increases in Nox2-derived radicals (see Figure 4) and reductions in expression of all 3 isoforms of SOD. Although increases in NAD(P)H oxidase are consistent with previous findings in hypercholesterolemic mice, reductions in SOD expression in our LDLr−/− mice contrasts with reports from ApoE−/− mice, where SOD expression increases during progression of atherosclerosis. Our mice, however, were exposed to prolonged hypercholesterolemia (6–12 months), compared with 3 to 4 months in other studies. Our findings may be explained by recent reports that suggest that older mice have an impaired ability to mount antioxidant responses to hyperlipidemic stressors.

If oxidative stress contributed to impaired vasomotor relaxation in this study, it might seem surprising that incubation of aortic rings with Tempo (an antioxidant) did not improve endothelial function, and even impaired vascular function in some groups. We have previously reported that Tempo can increase levels of hydrogen peroxide, which can subsequently induce production of endothelium-derived contracting factors. Although we focused on SOD expression in the current study, recent work also demonstrated adequate levels of catalase are critical for degradation of SOD-produced hydrogen peroxide, and subsequent atheroprotection with SOD1 overexpression. We do not have direct evidence for such a phenomenon in the current study, but it is an intriguing future direction to pursue in mice with hypercholesterolemia.

Reducing blood lipids after 6 months of hypercholesterolemia completely prevented progression of impairment in endothelial function from 6 to 12 months, and endothelial function in 12-month-old reversed mice was nearly identical to 12-month-old control mice. Furthermore, reducing blood lipids for only...
2 months after 12 months of hypercholesterolemia resulted in similar changes (see the online-only Data Supplement). In contrast to our hypothesis, however, these changes were not associated with reduction in expression of Nox2 or Nox4, but were instead more closely associated with increases in expression of SOD. Thus, lipid-lowering preserves endothelial function even after prolonged hypercholesterolemia, and may do so, in part, by increasing antioxidant defense mechanisms.

**Endothelium-Independent Relaxation**

As reported previously in hypercholesterolemic animals, impaired relaxation to acetylcholine after 12 months of...
hypercholesterolemia may be related, in part, to decreased nitric oxide bioavailability and to decreased responses to nitric oxide, as we observed reductions in relaxation to SNP. This observation is consistent with the concept that the nitric oxide–binding site of soluble guanylate cyclase can be oxidized by NAD(P)H-derived radicals, thereby reducing its responsiveness to nitric oxide and nitric oxide donors. If reductions in sensitivity to nitric oxide occur via this mechanism, however, our data suggest that acute treatment with an antioxidant (ie, Tempo) is not sufficient to reverse oxidation of soluble guanylate cyclase and improve responses to nitric oxide donors.

Reducing blood lipids also prevented impairment in responses to nitroprusside. Similar results were observed when blood lipids were reduced for 2 months after 12 months of hypercholesterolemia (see the online-only Data Supplement). These data suggest that sensitivity of vascular smooth muscle cell to nitric oxide is remarkably labile, even after a relatively short duration (2 months) of reduction in blood lipids.

**Tetrahydrobiopterin Deficiency Contributes to Endothelial Dysfunction With Hyperlipidemia**

After both 6 and 12 months of hypercholesterolemia, we found increased expression of GTPCH, but reduced expression of DHFR, which are enzymes related to the de novo and salvage pathways of tetrahydrobiopterin synthesis, respectively. These data are consistent with previous observations from ApoE−/− mice, in which GTPCH expression and activity were significantly increased after 5 months of severe hyperlipidemia and resulted in increased levels of vascular tetrahydrobiopterin. Incubation of vessels with sepiapterin (a DHFR-dependent BH4 precursor) at 6 months did not improve responses to acetylcholine. Treatment with sepiapterin, however, improved endothelium-dependent relaxation after 12 months of hyperlipidemia (Figure 5C). These data are consistent with studies that examined effects of administration of tetrahydrobiopterin to hypercholesterolemic mice (eg, orally for >2 weeks), where tetrahydrobiopterin reduces ROS uncoupling, attenuates inflammation, and improves endothelial function. Increasing tetrahydrobiopterin also improved endothelial function in patients with atherosclerosis. We speculate that, much like L-arginine (ie, the L-arginine paradox), tetrahydrobiopterin levels may need to exceed physiological levels by several fold in disease states to have a therapeutic benefit.

**Limitations**

In the current study, we used female mice for all experiments, as male LDLr−/−/ApoB100/100 mice tend to develop severe skin lesions after 10 to 12 months of Western diet feeding. By using only female animals, we were able to avoid changes in systemic inflammation secondary to the presence of large skin lesions in male mice, which are likely to be a major confounding variable when examining progression/regression of atherosclerosis.

We did not conduct quantitative measurements of superoxide levels in this study (eg, lucigenin-enhanced chemiluminescence), because most aortic tissue was used for studies of vascular function and gene expression, and because mice were treated for long periods of time (6–14 months).

We have not conducted extensive studies to examine cell type–specific changes in molecules during prolonged hypercholesterolemia. We recognize that the phenotypic consequence of altering expression of cholesterol transporters and numerous other enzymes may depend on the cell type in which the enzyme is expressed, and are deserving of mechanistic investigation in future studies.

**Conclusions**

Endothelium-dependent relaxation and sensitivity of smooth muscle cells to nitric oxide can be markedly improved after reduction of blood lipids in mice. These functional improvements appear to be a result, at least in part, of improvement in antioxidant defense mechanisms and de novo tetrahydrobiopterin production, but not reductions in NAD(P)H oxidase expression. Future studies examining regulation of these molecular changes may lead to novel therapeutic targets for treatment of endothelial dysfunction in atherosclerosis.

**Acknowledgments**

We thank Kathy Walters at the University of Iowa Central Microscopy Research Facility for help with immunostaining.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL062984 (D.D.H.), HL092235 (J.D.M.), NS024621 (D.D.H.), and funds from a Carver Research Trust Program of Excellence at the University of Iowa.
Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2013;33:459-465; originally published online January 10, 2013;
doi: 10.1161/ATVBAHA.112.252700

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure I. Atherosclerotic plaque area in aorta. Plaque size is markedly elevated in hypercholesterolemic mice at 6 and 12 months, and tends to be attenuated by reducing cholesterol levels (p = n.s. versus 12 month HCHOL). CTRL = normocholesterolemic group, HCHOL = hypercholesterolemic group, REV = “reversed”/regression group. * = p < 0.05 versus iso-time CTRL group.
Figure II. Macrophage levels (F4/80 immunostaining) in aorta. Macrophage levels are markedly elevated in hypercholesterolemic mice at 6 and 12 months (brown staining), and are dramatically attenuated by reducing cholesterol levels. CTRL = normocholesterolemic group, HCHOL = hypercholesterolemic group, REV = “reversed”/regression group. * = p < 0.05 versus iso-time CTRL group; # = p < 0.05 versus 6 month HCHOL group.
Figure III. Plaque fibrosis (Masson’s trichrome staining) in aorta. Aortic plaques in hypercholesterolemic mice at 6 and 12 months have lighter blue staining, indicating lower collagen content/fibrosis. Note that intensity of blue staining is markedly increased by reducing cholesterol levels, indicating an increase in plaque fibrosis and stability. CTRL = normocholesterolemic group, HCHOL = hypercholesterolemic group, REV = “reversed”/regression group.
Figure IV. Changes in endothelial function after incubation of vessel segments with a tetrahydrobiopterin precursor (Sepiapterin) in control and hypercholesterolemic at 12 months. Acute treatment with Sepiapterin does not improve responses to acetylcholine in normocholesterolemic mice, but improves maximal relaxation to acetylcholine in mice with prolonged, severe hypercholesterolemia.