An In Vitro Cynomolgus Vascular Surrogate System for Preclinical Drug Assessment and Human Translation


**Objectives**—The predictive value of animal and in vitro systems for drug development is limited, particularly for nonhuman primate studies as it is difficult to deduce the drug mechanism of action. We describe the development of an in vitro cynomolgus macaque vascular system that reflects the in vivo biology of healthy, atheroprone, or advanced inflammatory cardiovascular disease conditions.

**Approach and Results**—We compare the responses of the in vitro human and cynomolgus vascular systems to 4 statins. Although statins exert beneficial pleiotropic effects on the human vasculature, the mechanism of action is difficult to investigate at the tissue level. Using RNA sequencing, we quantified the response to statins and report that most statins significantly increased the expression of genes that promote vascular health while suppressing inflammatory cytokine gene expression. Applying computational pathway analytics, we identified statin-regulated biological themes, independent of cholesterol lowering, that provide mechanisms for off-target effects, including thrombosis, cell cycle regulation, glycogen metabolism, and ethanol degradation.

**Conclusions**—The cynomolgus vascular system described herein mimics the baseline and inflammatory regional biology of the human vasculature, including statin responsiveness, and provides mechanistic insight not achievable in vivo. *(Arterioscler Thromb Vasc Biol. 2015;35:2185-2195. DOI: 10.1161/ATVBAHA.115.306245.)*

**Key Words:** cell cycle ■ hemodynamics ■ hydroxymethylglutaryl-CoA reductase inhibitors ■ inflammation ■ *Macaca fascicularis*

Drug discovery involves initial target identification and validation for a disease with subsequent development of compounds to act on that target for disease intervention. Lead compounds are then optimized for safety and efficacy and undergo preclinical testing before entering clinical phase trials. Preclinical testing is important in predicting drug safety and efficacy in humans by performing validation trials in nonhuman animal models. These trials are costly, time consuming, and often involve multiple animal species, including nonhuman primates. Nonhuman primates may predict human drug response; however, their use is expensive and difficult. Moreover, when a negative result occurs, further experimentation to unmask the mechanism is considered unethical and is not warranted in nonhuman primates. Thus, the development of promising drugs is often prematurely terminated. The establishment of physiologically relevant in vitro animal model systems that provide strong predictive value is crucial for optimizing the drug development process. Here, we describe the development of an in vitro cynomolgus vascular surrogate system to aid in nonhuman primate studies.

The development of the cynomolgus vascular system was modeled after our well-characterized human vascular tissue system. This system mimics the in vivo endothelial and smooth muscle architecture, biology and physiology of the blood vessel wall, and is highly responsive to changes in hemodynamics, inflammatory milieu, and drug treatments. The vascular model has been configured to reflect an advanced inflammatory state characteristic of patients with atherosclerosis.

Cynomolgus macaques (*Macaca fascicularis*) are the most widely used nonhuman primates for atherosclerosis. Cynomolgus macaques are highly responsive to atherogenic diets, and the atherogenic response is influenced by lipoprotein diet composition. Atherogenic diet-fed cynomolgus macaques develop coronary artery lesions, raised plasma cholesterol levels, and lesions of similar composition to humans. Atherosclerosis development in cynomolgus macaques is highly influenced by genetic predisposition, confounding diseases (such as diabetes mellitus), exercise, sex (increased susceptibility in men), and behavioral and psychosocial factors.
All of these characteristics are similar to the human atherosclerosis disease, and thus, they make cynomolgus macaques a good nonhuman primate model for atherosclerosis.7,8

In this study, a cynomolgus vascular system was developed using primary endothelial cells (ECs), smooth muscle cells (SMCs), and hemodynamics derived in vivo from cynomolgus arteries. The system was validated against inflammatory risk factor response, including a comparison between human- and cynomolgus-derived oxidized low-density lipoproteins (LDLs), and exposure to several statins. A comparative analysis between the cynomolgus and human vascular tissue systems was conducted to determine the cross-species translational capabilities of these models, which is an important component in the transition from preclinical to human clinical studies.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Development of an In Vitro Cynomolgus Vascular Surrogate System

The human vascular surrogate system applies human-derived blood shear stress patterns to a transwell vascular coculture of ECs and SMCs (Figure 1A). Atherosclerotic lesions develop primarily at arterial sites of disturbed blood flow characterized by low time-averaged and oscillatory wall shear stress, often in bifurcated areas or regions of high curvature. Blood velocity profiles were previously obtained by magnetic resonance imaging from the human common carotid artery and the internal carotid sinus regions4 (Figure 1B). These measurements correlate with vascular regions that exhibit atheroprotective and atheroprone phenotypes, respectively.3 The cynomolgus vascular surrogate system applies the same correspondence of hemodynamic principles. Cynomolgus blood velocity profiles were acquired by ultrasound and wall shear stress values determined from the corresponding cynomolgus common carotid artery and the carotid bulb regions (Figure 1B).

Compared with human hemodynamic waveforms, there are several distinct differences in the profile patterns between cynomolgus and humans. In particular, the cynomolgus carotid bulb waveform exhibits reversal in blood flow with diminished pulsatile flow, whereas the human internal carotid sinus region mostly loses pulsatile flow (Figure 1B). The cynomolgus shear stress magnitude is much greater than the shear stress in humans (Table 1). Nevertheless, the cynomolgus common carotid and carotid bulb waveforms are used to mimic atheroprotective and atheroprone arterial regions in the cynomolgus macaque.7 The wall shear stress profiles are then applied to the respective cynomolgus and human vascular systems by way of a cone-and-plate viscometer that is incorporated into the EC and SMC transwell coculture setup (Figure 1A). Representative images of cynomolgus ECs and SMCs exposed to the cynomolgus atheroprotective waveform are shown (Figure 1A).

Initial characterization of the cynomolgus vascular surrogate system involved comparison with known hemodynamic effects imparted on human vascular cells. The cynomolgus and human vascular systems were exposed to atheroprotective and atheroprone hemodynamics for 48 hours, and gene expression patterns of the cynomolgus ECs and SMCs were determined by RNA sequencing. Initially, a panel of EC shear stress response genes was assessed in the presence or absence of hemodynamic flow, including genes important in promoting vascular endothelial health (kruppel-like factor 2 [KLF2], kruppel-like factor 4 [KLF4], endothelial nitric oxide synthase 3 [eNOS3], and heme oxygenase 1 [HMOX1]). eNOS is a potent vasodilator with strong inhibitory effects of platelet and monocyte activation and sustains vascular health by promoting antithrombotic and anti-inflammatory effects. The KLF2 and KLF4 transcription factors can positively regulate eNOS expression.9,10 HMOX1 is an oxidative stress response gene that has anti-inflammatory and vasoregulatory effects. The shear stress response is also characterized by downregulation of inflammatory and vasoconstrictive markers, such as C-X-C chemokine receptor type 4, chemokine ligand 2, angiopoietin 2, and endothelin 1 (EDN1). The analysis of shear stress activation of these genes demonstrates that the cynomolgus endothelial layer appropriately regulates these classical shear stress response genes when compared with samples not exposed to hemodynamics. An even greater effect was observed with the atheroprotective common carotid waveform (Figure 1C). Furthermore, we compared the fold changes in gene expression in ECs from our cynomolgus versus human vascular systems that were exposed to atherogenic hemodynamics from the common carotid and carotid bulb regions. We sought to determine similarities in gene expression fold change and directionality between the 2 systems when responding to changes in hemodynamics. To compare the effects of 2 different treatments, individual gene fold changes were plotted on the x and y axes. Overall, the expression of a large number of endothelial genes changed in the same direction when comparing the responses to hemodynamics of the cynomolgus and human vascular systems (Figure 1D, purple dots). However, in a relatively small subset of genes, the fold changes in expression were in opposite directions when comparing the 2 species.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
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<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>EDN1</td>
<td>endothelin 1</td>
</tr>
<tr>
<td>HMOX1</td>
<td>heme oxygenase 1</td>
</tr>
<tr>
<td>eNOS3</td>
<td>endothelial nitric oxide synthase 3</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>KLF2</td>
<td>kruppel-like factor 2</td>
</tr>
<tr>
<td>KLF4</td>
<td>kruppel-like factor 4</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>oxLDL</td>
<td>oxidized LDL</td>
</tr>
<tr>
<td>RSI</td>
<td>response similarity index</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular adhesion molecule 1</td>
</tr>
</tbody>
</table>

Nonstandard Abbreviations and Acronyms

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
Most of the endothelial genes changed in the same direction (response similarity index [RSI] > 0) in response to atherogenic hemodynamics when comparing the 2 species; in ECs, the ratio of genes with an RSI of >0.2 to genes with an RSI of <−0.2 was 4:1 (Figure I in the online-only Data Supplement).

Characterization of Cynomolgus- and Human-Derived Oxidized LDL

ECs exposed to atheroprone hemodynamics promote inflammation, monocyte transmigration and differentiation into macrophages, and subsequent atherosclerosis.11 Key components of the inflammatory milieu that predispose this region to atherosclerosis are tumor necrosis factor-α (TNF-α) and oxidized LDL (oxLDL). To mimic an advanced inflammatory state of atherogenesis, TNF-α and oxLDL were added to cells exposed to atheroprone hemodynamics. Because of the challenge of accessing large quantities of cynomolgus LDL, we tested whether cynomolgus-derived oxLDL or human-derived oxLDL would have a similar effect. Species-specific effects of oxLDL on the cynomolgus system were compared by transcriptomic analysis of RNA sequencing data and Western blot analysis.

Comparison of oxLDL- versus native LDL-mediated effects reveals that the overwhelming majority of genes in ECs and SMCs exhibit highly similar fold change and expression directionality between cynomolgus and human. A DEG with the same directionality in fold change for both species is represented in purple, whereas a DEG exhibiting opposite directionality is represented in green. The color intensity increases with statistical significance. The response similarity index (RSI) value is a single numeric value that incorporates the fold change and significance for each DEG in both species.

(Figure 1D, green dots). Most of the endothelial genes changed in the same direction (response similarity index [RSI]>0) in response to atherogenic hemodynamics when comparing the 2 species; in ECs, the ratio of genes with an RSI of >0.2 to genes with an RSI of <−0.2 was 4:1 (Figure I in the online-only Data Supplement).
endothelial NOS3 and smooth muscle ACTA2 (smooth muscle alpha-actin 2 gene) and KLF2 (Figure 2B). Cynomolgus- and human-derived oxLDL also upregulate oxidative stress–reducing genes (HMOX1 and superoxide dismutase 2), cell adhesion molecules (CAMs; E-selectin, intercellular adhesion molecule 1, and vascular adhesion molecule 1 [VCAM1]), and proinflammatory markers (interleukin [IL]-6, IL-8, chemokine ligand 2, and nuclear factor of κ light polypeptide gene enhancer in B-cells 1) in ECs and SMCs (Figure 2B). VCAM1 is likely upregulated by TNF-α and oxLDL in the intima of developing atherosclerotic plaques.12 ACTA2 encodes smooth muscle α-actin and is a marker of the smooth muscle contractile phenotype characterized by a quiescent, differentiated state. Downregulation of ACTA2 expression suggests that SMCs have lost their contractile phenotype and may have acquired a more proliferative, migratory phenotype.13 The cynomolgus-derived oxLDL-mediated increase in VCAM1 and decrease in ACTA2 gene expression were confirmed at the protein level by Western blot (Figure 2C).

**Statin Treatment of Cynomolgus and Human Vascular Systems**

Next, we exposed the cynomolgus vascular system to a well-characterized drug class and compared its response to the human system. Statins are a well-studied and ubiquitous class of drugs with proven efficacy for lipid-lowering and pleiotropic anti-inflammatory vascular effects. The cynomolgus and human vascular systems exposed to advanced inflammatory conditions were treated with 4 statins or the vehicle control. Rosuvastatin, atorvastatin, simvastatin, and cerivastatin were

![Figure 2](http://atvb.ahajournals.org/ Downloaded from

**Table 1. Blood Velocity Profiles Characterized From the Common Carotid and Carotid Bulb/Carotid Sinus Arterial Regions of the Cynomolgus Macaques and Humans**

<table>
<thead>
<tr>
<th></th>
<th>Cynomolgus</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Common</td>
<td>Bulb</td>
</tr>
<tr>
<td>Time average shear stress, dyne/cm²</td>
<td>27.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Maximum shear stress, dyne/cm²</td>
<td>88.7</td>
<td>50.2</td>
</tr>
<tr>
<td>Minimum shear stress, dyne/cm²</td>
<td>12.4</td>
<td>−16.4</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>
administered at the highest Food and Drug Administration–approved human $C_{\text{max}}$ concentration (Table 2). Global gene expression responses in statin-treated cynomolgus cells reveal that statins have the greatest response in ECs as observed by greater differentially expressed gene counts in ECs versus SMCs (Figure 3). Furthermore, cerivastatin exerted the maximal effect in both ECs and SMCs, with a greater response in SMCs (Figure 3). However, the rosuvastatin response was minimal (Figure 3).

Statins have been reported to exert beneficial pleiotropic effects on the vascular wall independent of cholesterol reduction. In the cynomolgus and human endothelium, statin treatment, except rosuvastatin, was found to increase NOS3, KLF2, and KLF4 and reduce cytokine IL-1B, IL-1A, IL-6, IL-8, chemokine (C-X-C motif) ligand 12, and EDN1 gene expression, supporting a healthier, less inflamed phenotype.
Gene expression for CAMs, E-selectin, VCAM1, and intercellular adhesion molecule 1, was not reduced, and in fact, it was upregulated by statins, except for simvastatin and cerivastatin in human ECs and rosuvastatin (Figure 4A). CAM P-selectin was also upregulated by all conditions, except rosuvastatin (Figure 4A). All statins, except rosuvastatin, also exerted some beneficial effects on the cyonolagus and human smooth muscle layer in the vascular systems, with a greater response in human (Figure 4B). EDN1 was downregulated, and it mediates smooth muscle vasoconstriction (Figure 4B). Furthermore, key genes of the coagulation pathway are differentially regulated by statins to promote an antithrombotic effect. Thrombomodulin is upregulated and inhibits thrombin, a major activator of coagulation; tissue-type plasminogen activator is upregulated and promotes fibrinolysis, and thus, it decreases thrombus formation; endothelial plasminogen activator inhibitor is downregulated and is an inhibitor of tissue-type plasminogen activator activity; coagulation factor 3 is downregulated and is a procoagulation factor (Figure 4B). Despite these beneficial effects, statin treatment reduced the gene expression of key markers of smooth muscle contractility, including ACTA2, caldesmon 1, myosin light chain kinase, transgelin, and myocardin (Figure 4B). Increased KLF4 expression in the SMCs can also promote loss of the differentiated SMC contractile phenotype (Figure 4B).

Aside from anticipated vascular-specific effects imparted by statins, a pathway-weighting approach identified several biological themes that are not specific to the vasculature. These include cell cycle regulation, ethanol degradation, and glycogen metabolism. Cyonolagus- and human-derived oXLDL induced a global downregulation of many cell cycle regulatory genes, including cyclins and minichromosome maintenance proteins, whereas cyclin-dependent kinase inhibitors were upregulated (Figure 5A). This effect was further enhanced by statin treatment, except for rosuvastatin (Figure 5A). Global downregulation of cell cycle regulatory proteins was more pronounced in ECs (Figure 5A). Proliferating cell nuclear antigen protein was also downregulated in cyonolagus ECs and SMCs by oXLDL, consistent with reduced cell cycle gene regulation by oXLDL (Figure 5B). No significant changes were observed for growth arrest and DNA damage 45 protein levels by oXLDL (Figure 5B); growth arrest and DNA damage 45 is induced by DNA damage to activate the G2/M checkpoint and subsequent growth suppression. Furthermore, ethanol metabolism generates many toxic acetaldehyde byproducts that must be further metabolized by acetaldehyde dehydrogenases to prevent their accumulation. Acetaldehyde dehydrogenases belong to the aldehyde dehydrogenase (ALDH) family and include ALDH1A1, ALDH2, and ALDH1B1. Although ALDH2 is the main enzyme that metabolizes acetaldehydes, ALDH1A1 and ALDH1B1 can also act on these byproducts. We observed that oXLDL and statins downregulated ALDH1A1 and ALDH1B1 under many conditions, which may promote acetaldehyde accumulation (Figure 5C).

Finally, many genes important in glycogen regulation and metabolism were also found to be downregulated by statin treatment in cyonolagus and human vascular cells, with the exception of rosuvastatin that elicited no changes and cerivastatin that upregulated phosphorylase, glycogen, brain, phosphoglycerate mutase 2, and phosphoenolpyruvate carboxykinase 2 in human SMCs and phosphoenolpyruvate carboxykinase 2 in cyonolagus SMCs (Figure 6). Perturbation of gene expression of this pathway will impair glycogen regulation and may lead to inefficient glucose utilization and storage.

**Discussion**

We describe the successful development of a cyonolagus vascular surrogate system that recapitulates expected biology observed in the vascular wall. The cyonolagus vascular system responds to natural arterial shear stress patterns from the common carotid and carotid bulb regions in a manner that reflects the atheroprotective and atheroprone conditions, respectively. Precise regional shear stress activates classical shear stress atheroprotective responsive genes in the vascular endothelium, including NOS3, KLF2, KLF4, and HMOX1. These hemodynamics also reduce the expression of proinflammatory cytokines, vasoregulatory proteins, and genes that disrupt EC barriers, such as chemokine ligand 2, EDN1, C-X-C chemokine receptor type 4, and angiotensin 2. Thus, the cyonolagus vascular endothelium, when in direct contact with local hemodynamics, responds in a way that closely correlates with the human vascular system.

Comparison of cyonolagus- versus human-derived oXLDL is important in assessing differences in species-specific effects of LDL. Similar to humans, the cyonolagus macaque is highly responsive to elevated dietary cholesterol levels, leading to increased serum lipids and altered lipid composition. Compared with other nonhuman primates, cyonolagus...
LDL exhibits increased atherogenic potential because of its larger, polydisperse LDL and increased apolipoprotein E fraction. This larger size will increase the amount of cholesterol ester per LDL particle delivered to cells, increasing the rate of cholesterol ester accumulation and plaque development. The metabolic heterogeneity of responses induced by cynomolgus- versus human-derived oxLDL can in large part be predicted by the heterogeneous composition of elevated LDL. Assessing the differences in oxLDL composition will be useful in identifying metabolic and genetic predictors for responses to dietary cholesterol and how responses between the systems can be compared. Our studies reveal that cynomolgus- and human-derived oxLDL elicit nearly identical responses in the cynomolgus vascular wall under advanced inflammatory conditions, as measured by high overlap of differentially expressed gene response. These similarities suggest that human LDL may be substituted for studies when cynomolgus LDL is scarce.

Statin-mediated responses in the cynomolgus vascular system were compared with our human surrogate system. Cerivastatin elicited the most robust response, whereas rosuvastatin had little-to-no effect (Figure 3). Cerivastatin exhibits the greatest bioavailability at 60% compared with 14% and <5% for atorvastatin and simvastatin, respectively. Cerivastatin, along with atorvastatin and simvastatin, is a lipophilic compound that can passively diffuse through the cell membrane, whereas rosuvastatin is hydrophilic and requires active transport. The ABC and SLC gene family transporters are responsible for carrier-mediated uptake of statins and are primarily hepatic-specific markers. Thus, decreased expression of these transporters in the vascular wall will blunt rosuvastatin-mediated effects, supporting the idea that hydrophilic statins are less effective intracellular agents. The minimal rosuvastatin-mediated response is also consistent with reported lack of lipid-lowering effects by rosuvastatin in cynomolgus macaques. Thus, the observed statin response is consistent with the physiochemical properties of each statin and was consistent between cynomolgus and humans.

Statin treatment, particularly with the lipophilic statins, was able to reverse many of the detrimental effects induced by advanced inflammatory conditions. Endothelial proinflammatory cytokines IL-1A, IL-6, and IL-8 were downregulated by...
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This is consistent with reported decreases of serum IL-6, IL-8, and CXCL12 in human patients. In addition, key genes (NOS3, KLF2, and KLF4) for maintaining EC health were upregulated by statins, whereas EDN1 was downregulated. Statins improve arterial dilatory function in cynomolgus macaques and humans, in part, through regulating eNOS, KLF2, and KLF4 expression. Finally, statins promote antithrombotic effects, manifesting in this study as decreased prothrombotic (coagulation factor 3 and endothelial plasminogen activator inhibitor) and increased antithrombotic (thrombomodulin and tissue-type plasminogen activator) gene expression. The beneficial effects exerted by statin treatment are thought to be independent of lipid-lowering effects given that these changes are observed acutely before lipid changes.

**Table 3. Summary of Gene Mutations That Lead to Muscle Pathologies and the Observed Statin-Induced Gene Expression of Those Genes in the Cynomolgus and Human Vascular Surrogate Systems**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease</th>
<th>Symptoms</th>
<th>Statin Effect (Cyno)</th>
<th>Statin Effect (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDOA</td>
<td>GSD12 (ALDOA deficiency)</td>
<td>Nonspherocytic anemia, myopathy, rhabdomyolysis</td>
<td>↓Simvastatin</td>
<td>↓Simvastatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓Cerivastatin</td>
<td>↓Cerivastatin</td>
</tr>
<tr>
<td>LDHA</td>
<td>GSD11 (LDHA deficiency)</td>
<td>Fatigue, myoglobinuria, muscle stiffness, cramps, exertional myoglobinuria</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>GYS1</td>
<td>GSD0 (GYS1 deficiency)</td>
<td>Exercise intolerance, cardiomyopathy, muscle weakness, lack of muscle glycogen</td>
<td>↓Simvastatin</td>
<td>↓Atorvastatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓Cerivastatin</td>
<td>↓Cerivastatin</td>
</tr>
<tr>
<td>PGM1</td>
<td>GSD14 (PGM1 deficiency)</td>
<td>Exercise intolerance, episodes of rhabdomyolysis</td>
<td>↓Simvastatin</td>
<td>↓Atorvastatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓Cerivastatin</td>
<td>↓Cerivastatin</td>
</tr>
<tr>
<td>PYGB</td>
<td>*Increased plasma levels</td>
<td>Hypertrophic cardiomyopathy</td>
<td>NE</td>
<td>↑Cerivastatin</td>
</tr>
</tbody>
</table>

Cyno indicates cynomolgus; EC, endothelial cell; NE, no statin effect; and SMC, smooth muscle cell.

**Figure 6.** Statins may impair glycogen metabolism/glycolysis by downregulating genes of this pathway. The cynomolgus (Cyno) and human vascular surrogate systems were exposed to baseline atheroprone (10 μg/mL of Cyno-derived native low-density lipoprotein [nLDL] or 50 μg/mL of human-derived nLDL, respectively) or advanced inflammatory conditions (10 μg/mL of Cyno-derived oxidized LDL [oxLDL] + 0.05 ng/mL of tumor necrosis factor-α [TNF-α] or 50 μg/mL of human-derived oxLDL + 0.05 ng/mL of TNF-α, respectively) with either vehicle control or statin treatment. Statin-mediated regulation of gene expression of key genes of the glycogen metabolism/glycolysis pathway is represented in the heatmap. Gene expression is represented on the heatmaps as log2 fold (red, upregulation; blue, downregulation). A schematic of the pathway and key genes for glycogen metabolism, glycolysis, and gluconeogenesis is represented. EC indicates endothelial cell; and SMC, smooth muscle cell.
As a consequence, independent of lipid lowering, statins may improve (1) endothelial function via increased nitric oxide production (due to increased NOS3), (2) anti-inflammatory effects due to reduction of acute phase proteins, (3) antioxidant effects due to increased scavenging of superoxide and inhibition of superoxide generators, (4) antithrombotic effects that promote fibrinolysis and decrease platelet aggregation, and (5) stabilization of atherosclerotic plaque.

Unexpectedly, gene expression for CAMs, E-selectin, VCAM1, and intercellular adhesion molecule 1, was not reduced and in fact was upregulated by statin treatment under most conditions. This effect is consistent with the lack of in vivo statin-mediated reduction on circulating E-selectin, VCAM1, and intercellular adhesion molecule 1 expression in patients with hypercholesterolemia.\(^{31,32}\) Although the reason for this lack of CAM reduction is not clear, it may be a function of persistent oxLDL in the circulating serum that may directly act to upregulate CAMs. oxLDL can upregulate CAM expression in a TNF-α-dependent manner.\(^{33,34}\) In fact, TNF-α genes are upregulated under conditions with elevated CAM expression despite decreased expression of cytokine expression (data not shown). However, maintained lipid reduction may lead to reduced CAM expression. Furthermore, markers of SMC contractility were decreased. Although the SMC contractile phenotype is important in maintaining cell quiescence, statins are known to decrease these markers, such as myocardin, a global transcriptional regulator of SMC markers, and prevent vascular contraction.\(^{35,36}\) Coupled with statin-mediated NOS3 increase, this can contribute to reported statin-mediated vasodilatory effects.

We also demonstrated that the cynomolgus vascular surrogate system responds to statin treatment consistent with positive statin-mediated pleiotropic effects observed in cynomolgus in vivo, as well as human patients, and in the human vascular system. Clinical evidence suggests that statin treatment is associated with antiproliferative and antimetastatic properties.\(^{37}\) Statin inhibition of hydroxymethylglutaryl CoA reductase results in inhibition of the cholesterol biosynthetic pathway. Mevalonate is a precursor of cholesterol and other lipid products, many of which regulate cell cycle, such as dolichol, geranylpyrophosphate, and farnesylpyrophosphate. Dolichol stimulates DNA synthesis, whereas geranylpyrophosphate and farnesylpyrophosphate cause isoprenylation of intracellular Ras and Rho G-proteins, important signaling molecules that influence gene transcription of cell cycle regulatory proteins. In addition to lipid-lowering effects mediating reduced cell proliferation, we observed that cyclins and minichromosome maintenance proteins are downregulated, whereas cyclin-dependent kinase inhibitors are upregulated. In fact, consistent with reports demonstrating that cell cycle kinase inhibitors p21 and p27 are stabilized, thereby arresting cells in the G1 phase of the cell cycle,\(^{37}\) we observed that the genes cyclin-dependent kinase inhibitor 1A and cyclin-dependent kinase inhibitor 1B for these kinase inhibitors, respectively, are upregulated. Interestingly, statins alone may not be effective anticancer agents; when combined with other cytotoxic agents, data suggest an improvement in chemotherapeutic effects.\(^{37}\) The cynomolgus and human vascular systems provide a useful platform in which dual therapy regimens can be easily tested for safety and efficacy while elucidating the drug mechanism of action.

The cynomolgus and human vascular systems were invaluable in providing mechanistic insight into the pleiotropic vascular effects independent of cholesterol lowering by statins. Computational analysis of the differentially expressed genes revealed multiple common biological pathways regulated by each statin treatment that yielded clues to mechanisms for observed off-target effects. Cevirastatin was withdrawn from the market because of high reported incidences of rhabdomyolysis, a condition characterized by breakdown of muscle tissue and release of myoglobin into blood. Myoglobinuria can cause renal damage and is potentially fatal. Thus, patients taking statins are warned to immediately discontinue use if any symptom of muscle pain or weakness is experienced. Consistent with this off-target effect, we observed that many genes regulating glycogen metabolism were downregulated by all statins, except rosuvastatin, in cynomolgus and human cells. Carbohydrate utilization from intramuscular glycogen stores via glycogenolysis is necessary to provide the necessary energy for working muscles. If enzymes regulating this process are low or absent, myopathy and eventual rhabdomyolysis can result. Altered regulation of many genes regulating glycogen metabolism is associated with symptoms of myopathy (Table 3). We observed that statin treatment in the cynomolgus and human vascular systems alters the expression level of these genes in a manner that is consistent with myopathy (Table 3; Figure 6). In particular, cerivastatin exhibits the most robust response of genes implicated in myopathies, consistent with its clinical incidence of rhabdomyolysis.

Altered glycogen regulation/metabolism has additional effects on cellular function. Statin treatment has been associated with a modest risk for type 2 diabetes mellitus development.\(^{39}\) Type 2 diabetes mellitus is marked by impaired insulin-stimulated glucose uptake, which reduces insulin-stimulated muscle glycogen synthesis. Thus, statin-mediated defects in glycogen regulation may induce or further exacerbate diabetes mellitus symptoms. In addition, increased glucose metabolism is a hallmark of cancer. Reduction of glycogen metabolism genes could lead to decreased glucose metabolism and thus decreased cell proliferation. Indeed, decreased expression of hexokinase 2 and glucose-6-phosphate isomerase genes has been associated with reduced cell growth.\(^{39,41}\) We observed that these genes are also downregulated by statin treatment, leading to reduced glucose metabolism and consequent reduced cell proliferation, consistent with anticancer effects reported with statin treatment.

Finally, we detected that the acetaldehyde dehydrogenase genes, ALDH1A1 and ALDH1B1, were downregulated by statin treatment. These enzymes are important in the ethanol degradation process whereby alcohol dehydrogenase oxidizes ethanol in the liver into acetaldehyde, which is then oxidized by acetaldehyde dehydrogenases into nontoxic acetic acid. However, the loss of acetaldehyde dehydrogenase activity will lead to accumulation of acetaldehyde and DNA damage. Acetaldehyde accumulation can induce alcoholic myopathy via its effect on decreasing protein synthesis, increasing
proteolysis, and generating acetaldehyde protein adducts\(^{42}\) and is an additional potential mechanism for statin-induced rhabdomyolysis. Interestingly, the aldehyde dehydrogenase ALDH3A1 expression is increased by statins and can inhibit cell proliferation in human corneal epithelial cells,\(^{43}\) thus representing another mechanism for statin-mediated anticancer effects.

We describe here the development of a cynomolgus vascular surrogate system that mimics the baseline and inflammatory regional biology, as well as statin responsiveness, of the human vascular surrogate system. These systems enable mechanistic insight not achievable with in vivo studies. The overwhelming similarities between the cynomolgus and human vascular systems indicate that these platforms will provide significant translatability between nonhuman primate and human studies. Although we did not validate the translational statin response in the in vivo cynomolgus macaque because of time and cost constraints, we did perform a similar study where we validated the translation drug response of fenoldopam in rats with our rat vascular surrogate system and demonstrated similar responses.\(^{44–46}\) Finally, we have found that certain biological pathways that may explain statin effects on other tissues, such as skeletal muscle, are conserved in our vascular tissue. Thus, many of the effects observed in the vascular system potentially may be translated to other organ systems.

**Acknowledgments**

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**Disclosures**

None.

**References**


**Significance**

There is an unmet need to improve the predictive value of animal and in vitro systems for drug development, particularly in deducing the drug mechanism of action in nonhuman primate studies. We describe the development of an in vitro cynomolgus macaque vascular system that reflects the in vivo biology of healthy, atheropone, or advanced inflammatory cardiovascular disease conditions and determine whether statin responsiveness is similar to humans. We report that most statins significantly increased the expression of genes that promote vascular health while suppressing inflammatory cytokine gene expression, in agreement with known positive pleiotropic effects of statins. We identified statin-regulated biological themes independent of cholesterol lowering that provide mechanisms for off-target effects, including myopathy and diabetes mellitus. The cynomolgus vascular system described herein mimics baseline and inflammatory regional biology of the human vasculature, including statin-responsiveness, and provides mechanistic insight not achievable in vivo.
An In Vitro Cynomolgus Vascular Surrogate System for Preclinical Drug Assessment and Human Translation

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MATERIALS AND METHODS

An in vitro Cynomolgus Vascular Surrogate System for Preclinical Drug Assessment and Human Translation

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Detailed Materials and Methods:

**Cell Isolation and Culture**

Primary endothelial cells (ECs) and smooth muscles cells (SMCs) of human aortic origin (primarily from ascending aorta through the aortic arch) from 5 different donors were purchased from Lonza (USA) and tested separately in experiments (Human ECs: donor 1 = 43yr old male, donor 2 = 27yr old male, donor 3 = 59yr old male, donor 4 = 24yr old female; Human SMCs: donor 1 = 21yr old male, donor 2 = 20yr old male, donor 3 = 36yr old male, donor 4 = 20yr old male). Cynomolgus primary ECs and SMCs were isolated from the aortic arch through the descending thoracic aorta of aortic arteries of cynomolgus macaque (tissue provided by SNBL in cooperation with Amgen). ECs (from a single 5.5yr old cynomolgus female donor) were isolated and cultured following digestion using 0.2% type I collagenase. Isolated cells were incubated with 10µg/ml DiI-Ac-LDL (acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl – 3,3',3',3'-tetramethyl-indocarbocyanine perchlorate; Biomedical Technologies BT-902) for 4 hours, trypsinized, and sorted by flow cytometry (Supplemental Figure III). DiI-Ac-LDL binds both ECs and macrophages, however, ECs can be preferentially sorted due to decreased labeling compared to macrophages. Pure populations of ECs were collected and expanded for these studies. SMCs were isolated from the cynomolgus aorta of a separate 7.5yr old female donor by Lonza (USA). Purified SMCs from this isolation were expanded and used for these studies. ECs and SMCs were used up to passage 6 and 10, respectively, which has been established to retain the basal EC/SMC phenotype. Human and cynomolgus ECs and SMCs were maintained in EGM-2 BulletKit (Lonza CC-3162) and SmGM-2 BulletKit (Lonza CC-3182) media, respectively, except EGM-2 BulletKit media for cynomolgus cells supplemented with 10% FBS.

**Cynomolgus- and Human-Derived LDL**

Human native LDL (nLDL) was purchased from Kalen Biomedical (770200). Collecting cynomolgus LDL involved feeding ten cynomolgus macaques (housed at the Wake Forest Center for Comparative Medicine, Winston-Salem NC) an atherogenic diet (a custom formulated Typical American Diet that consists of 36% fat, 18% protein (casein-based), 46% carbohydrates, and 0.16mg/kcal cholesterol; see reference (1) for details) for 9 weeks to elevate circulating LDL levels. Three blood draws were performed at weeks 7, 8, and 9, pooled, and LDL isolated by ultracentrifugation. LDL was isolated by a standard procedure at Wake Forest. Briefly, pooled plasma was adjusted to 1.019 g/ml, spun at 50,000rpm at 15°C for 18 hours. The bottom crude fraction containing LDL + HDL particles was collected, density adjusted to 1.063g/ml, and spun again. The top crude fraction of LDL was collected and dialyzed in PBS. Protein and cholesterol quantity were determined by the Lowry method and a cholesterol enzymatic assay, respectively. Finally, using a modified protocol adapted from reference (2), human and cynomolgus nLDL was oxidized via the following procedure: nLDL was dialyzed overnight in PBS to remove EDTA. Next, nLDL was dialyzed in PBS containing 13.8µM CuSO₄ for three days to oxidize the LDL. Following completion of oxidation, LDL was dialyzed in PBS containing 50µM EDTA to remove
excess Cu. Electrophoretic migration of oxidized LDL (oxLDL) versus nLDL provides a measure of the oxidative state of LDL.

**Transwell Co-Culture Plating Conditions and Hemodynamic Exposure**

The transwell co-culture plating and hemodynamic flow device setup is explained in detail in reference (3) and Figure 1A. In brief, a porous polycarbonate transwell membrane (Corning Inc.) was coated with 0.1% gelatin on the top and bottom surface of the membrane. Human or cynomolgus SMCs were plated on the bottom transwell surface and human or cynomolgus ECs were plated on the top transwell surface. Transwells plated with cells were placed in flow media containing reduced serum (M199 supplemented with 2% FBS, 2mM L-glutamine, and 100U/ml penicillin-streptomycin) for flow experiments. However, flow media for cynomolgus ECs was supplemented with 10% EGM-2 media.

The co-cultured ECs and SMCs were then exposed to physiologically relevant hemodynamics utilizing a direct drive cone-and-plate viscometer described in reference (3). Flow media was prepared with dextran to obtain the desired viscosity necessary for transmitting physiological species- and waveform-specific shear forces from motor/cone assembly to the cells. Hemodynamic flow was imparted by the rotating cone to the EC layer of the EC-SMC co-culture transwell setup. Human hemodynamic shear stress profiles were derived from the human common carotid artery and internal carotid sinus, areas of atheroprotective/healthy regions or areas prone to atherosclerosis (atheroprone, as previously described in reference (4) and Figure 1B). Similarly, cynomolgus hemodynamic velocity profiles were obtained from the cynomolgus atheroprotective-like common carotid artery or the atheroprone-like carotid bulb region by ultrasound at the Wake Forest Center for Comparative Medicine, Winston-Salem NC (Figure 1B). Wall shear stress values were calculated from the blood velocity profiles and applied in the co-culture transwell cone-and-plate device (Figure 1A).

**Normal and Pathological Hemodynamic Conditions and Drug Treatments**

After human and cynomolgus cells were exposed to baseline atheroprotective or atheroprone hemodynamics for 18 hours, cells were exposed to species-specific nLDL or oxLDL plus human recombinant tumor necrosis factor-α (TNFα; 0.05ng/ml; R&D Systems 210-TA-010) in the atheroprone condition for an additional 30 hours. Human or cynomolgus LDL was added at 10µg/ml in cynomolgus cells and human LDL at 50µg/ml in human cells, as indicated in each experiment. The addition of oxLDL and TNFα in the atheroprone state mimics advanced inflammatory conditions in the context of cardiovascular disease.

For statin studies, cells exposed to advanced inflammatory conditions were treated at the 24 hour timepoint with one of four different statin drugs or a vehicle control (dimethyl sulfoxide, DMSO) for an additional 24 hours. Concentrations were calculated to approximate the maximum steady-state plasma levels (Cmax-ss) attained following human multi-dose exposure at the highest FDA-approved therapeutic dose, also taking into account the occurrence of any pharmacologically active metabolites. The concentrations selected were: atorvastatin, 457nM; cerivastatin, 58nM; simvastatin, 297nM; and rosuvastatin, 74nM (5). All drugs were prepared in DMSO and diluted 1:1000 in the experiment to achieve the final experimental concentration. The
final concentration of DMSO is all experiments was 0.1%.
All experiments were terminated after 48 hours and cells harvested for RNA or protein expression studies.

**Confocal Microscopy**
Cynomolgus endothelial and smooth muscle cells plated on transwells and exposed to hemodynamics were fixed in 4% paraformaldehyde, permeabilized with 0.2% triton-X 100 for 10 min., and stained with the following antibodies: Cy3-conjugated smooth muscle alpha actin (Sigma C6198) and Alexa Fluor 488-conjugated phalloidin (Life Technologies A12379). Fluorescence images were acquired with a Nikon 20X, 0.5 numerical aperture objective lens on a Nikon C1 confocal microscope with EZ-C1 v3.9 software.

**Western Blot Analysis**
Following 48 hours of cell exposure to hemodynamics, transwells containing ECs and SMCs were rinsed with cold PBS containing Ca^{2+}/Mg^{2+} and ECs and SMCs were separately collected as described in reference (1). Cell pellets were lysed in 2X Laemmlli sample buffer (BioRad 161-0737) containing 350mM dithiothreitol. Total protein lysates were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose. Blots were probed overnight with primary antibody and 30 min. at room temperature with secondary antibody in blocking buffer (LI-COR 927-40000). Primary antibodies (1:1000) include: β-actin (Sigma A1978), tubulin (Abcam ab173831), ACTA2 (Pierce MA5-15806), VCAM (R&D Systems BBA19), PCNA (Abcam ab18197), and GADD45α (Cell Signaling 4632S). Secondary antibodies (1:15,000) include: IRDye 680LT Donkey Anti-Mouse (LI-COR 926-68022), IRDye 800CW Donkey Anti-Mouse (LI-COR 926-32212), IRDye 680LT Donkey Anti-Rabbit (LI-COR 926-68023), IRDye 800CW Donkey Anti-Rabbit (LI-COR 926-32213), IRDye 680LT Donkey Anti-Goat (LI-COR 926-68024), and IRDye 800CW Donkey Anti-Goat (LI-COR 926-32214). The LI-COR Odyssey infrared imager was used for image acquisition and the LI-COR Odyssey Image Studio software used for densitometry analysis.

**RNA Preparation and RNA Deep Sequencing**
EC and SMC cell pellets were collected and RNA isolated using the Invitrogen Purelink RNA Mini kit (12183018A) according to manufacturer’s instructions. RNA concentration was determined with the Nanodrop and RNA integrity was determined using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 kit (5067-1511) according to manufacturer’s instructions. Samples with a minimum RNA Integrity Number 7.0 were used for further transcriptomics processing. 1µg RNA per sample was submitted to Expression Analysis, Inc. for Illumina-based RNA deep sequencing. Approximately 20 million 50 base paired-end reads were generated per sample. For each treatment, 4 EC and 4 SMC samples of Cynomolgous macaque (all same donor) and 5 EC and 5 SMC samples of human (each different donor) were run.

**Data Analysis and Statistics**
Following RNA deep sequencing, the cynomolgus reads were aligned to the rhesus macaque (Macaca mulatta) transcriptome (genome build mmul1). Human reads
were aligned to a standard hg19 human transcriptome. Alignment was done using Bowtie 0.12.9 with the final gene counts calculated using RSEM 1.2.0. Orthologs between rhesus and human were determined using Ensembl 1-to-1 orthologs from build 69.

Three RNA deep sequencing quality control measurements were performed to detect outliers and batch effects associated with the dataset: 1) false color heatmaps of distance between samples, 2) principal component analyses, and 3) overlapping density estimates. After evaluating these quality control outputs, outliers were excluded and batch effects, which consisted of human donor variability, removed. The RNA deep sequencing dataset counts were then analyzed to determine differentially expressed genes (DEGs). Methods for DEG determination were implemented using the open source R/BioConductor software (http://www.bioconductor.org) and the edgeR package. Only genes that passed a threshold of more than 2 counts per million in at least 4 cynomolgus samples or 5 human samples were included for further analysis. A gene was determined to be a DEG by passing an FDR threshold of 10% (using the Benjamini & Hochberg FDR correction). Select genes of relevant biological processes were chosen to build response heatmaps that depict changes in gene expression based on a log2fold scale of -2 to 2: blue = downregulation, red = upregulation, white = no change; the intensity of the color reflects the magnitude of change; a white dot indicates a statistically significant change in the expression of the corresponding gene (FDR<10%). To compare the effects of two different treatments or conditions, individual gene fold-changes were plotted on x- and y-axes. If two conditions stimulated identical responses, all the data would fall on the identity line of a gene-by-gene scatterplot. The Response Similarity Index (RSI) was calculated for each gene and determines the degree to which the two conditions alter gene expression in the same (RSI>0.2, purple dots) or opposite (RSI<-0.2, green dots) manner.

For western analyses, student’s t-test was performed to determine statistically significant differences in protein expression levels.
References:


SUPPLEMENTARY MATERIAL

An *in vitro* Cynomolgus Vascular Surrogate System for Preclinical Drug Assessment and Human Translation

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Supplemental Figures and Figure Legends:

Supplemental Figure I.
Comparison of carotid bulb and common carotid hemodynamics on cynomologus and human endothelial cells reveal an overall similarity in gene response. The cynomolgus and human vascular surrogate systems were exposed to hemodynamics from the atheroprotective carotid bulb and atheroprone common carotid arterial regions for 48 hours and endothelial and smooth muscle cells were processed for RNA sequencing. A histogram analysis of the genes from the scatterplot in Figure 1D represents cynomolgus versus human endothelial cell genes when comparing carotid bulb versus common carotid hemodynamic effects for each species. Each bar is composed of genes that exhibit similar or opposing log2fold changes. Bars composed of genes with similar directionality in fold-change for both species are represented in purple. Bars composed of genes with opposite directionality are represented in green. The color intensity increases with statistical significance. The response similarity index (RSI) is a single numerical value that incorporates the fold-change and significance for each gene in both species. Comparison of carotid bulb- versus common carotid-mediated effects reveals that a majority of the genes in endothelial cells exhibit similar fold-change and expression directionality between cynomolgus and human, as evidenced by a large number of genes (n=1905) with RSI>0.2. Opposite regulation is also apparent in a smaller subset of genes (n=475) with RSI <-0.2.
Supplemental Figure II. **Cynomologus- and human-derived oxLDL induce strongly similar gene expression changes in endothelial and smooth muscle cells.** The cynomolgus vascular surrogate system was exposed to atheroprone carotid bulb hemodynamics for 18 hours and then to baseline atheroprone or advanced inflammatory conditions for an additional 30 hours. The baseline atheroprone condition is achieved by adding cynomolgus- or human-derived nLDL (10µg/ml) while the advanced inflammatory condition is achieved by adding TNFα (0.05ng/ml) and cynomolgus- or human-derived oxLDL (10µg/ml). A histogram analysis of the genes from the scatterplots in Figure 2A represents cynomolgus endothelial and smooth muscle cell genes comparing oxLDL + TNFα versus nLDL response when LDL is of cynomolgus or human origin. Each bar is composed of genes that exhibit similar or opposing log2fold changes. Bars composed of genes with similar directionality in fold-change for both species are represented in purple. Bars composed of genes with opposite directionality are represented in green. The color intensity increases with statistical significance. The response similarity index (RSI) is a single numerical value that incorporates the fold-change and significance of being a DEG for each gene in both species. Comparison of oxLDL- versus nLDL-mediated effects reveals that most of the genes in endothelial and smooth muscle cells exhibit overwhelmingly similar fold-change and expression directionality between cynomolgus- and human-derived oxLDL and that this similarity of expression is associated with strong significance given the greater distribution of bars near RSI=1. In ECs, gene expression exhibited highly similar fold change and directionality, as evidenced by 3930 genes with RSI>0.2 and only 43 genes with RSI <-0.2. Similarly, in SMCs there were 3696 genes with RSI>0.2 and only 76 genes with RSI <-0.2.

Supplemental Figure II
Supplemental Figure III.

**Fluorescence activated cell sorting (FACs) of endothelial cells from cynomolgus aorta.** Endothelial cells were isolated from the aortic artery of a cynomolgus macaque. Cells were incubated with 10µg/ml Dil-Ac-LDL and sorted by flow cytometry by size exclusion and PE- signal detection to isolate endothelial cells. Two sequential purifications were performed to prepare a homogenous population of cynomolgus endothelial cells.

Supplemental Figure III