Hepatic Lipase Deficiency Delays Atherosclerosis, Myocardial Infarction, and Cardiac Dysfunction and Extends Lifespan in SR-BI/Apolipoprotein E Double Knockout Mice

Sharon L. Karackattu, Bernardo Trigatti, Monty Krieger

Objective—SR-BI/apolipoprotein (apo) E double knockout (dKO) mice exhibit many features of human coronary heart disease (CHD), including occlusive coronary atherosclerosis, cardiac hypertrophy, myocardial infarctions, and premature death. Here we determined the effects on this pathology of hepatic lipase (HL) deficiency, which has been shown to significantly modulate atherosclerosis.

Method and Results—The SR-BI/apolipoprotein (apo) E triple knockout (tKO) mice generated for this study lived significantly longer (37%) than corresponding dKO controls (average lifespans: 63.0±0.8 versus 46.0±0.3 days), despite their increased plasma cholesterol levels. At 6 weeks of age, compared with dKO mice, tKOs exhibited significantly less aortic root and coronary artery occlusive atherosclerosis, and improved cardiac structure and function. However, by 9 weeks of age the hearts of tKO mice exhibited lipid-rich coronary occlusions, myocardial infarctions, and cardiac dysfunction essentially identical to that of 6-week-old dKO mice.

Conclusions—HL-deficiency delays the onset and/or progression of atherosclerosis via a SR-BI–independent mechanism. Extent of occlusive coronary arterial lesions was more closely associated with cardiac dysfunction and lifespan than the amount of aortic root atherosclerosis, suggesting that these occlusions in dKO mice are responsible for ischemia, myocardial infarctions, and premature death. (Arterioscler Thromb Vasc Biol. 2006;26:548-554.)

Key Words: atherosclerosis  ■  hepatic lipase  ■  high density lipoprotein receptor  ■  myocardial infarction

Though apolipoprotein E (apoE) or low-density lipoprotein receptor (LDLR) knockout (KO) murine models of dyslipidemia are often used to study lipoprotein metabolism and atherosclerosis,1 they usually do not exhibit spontaneous occlusive coronary artery disease, myocardial infarction (MI), cardiac dysfunction and premature death, hallmarks of human coronary heart disease (CHD). Double knockout (dKO) mice deficient in the high-density lipoprotein (HDL) receptor (scavenger receptor class B type I, SR-BI) and apoE exhibit extensive aortic sinus atherosclerosis (advanced plaques with fibrous caps2 that contain macrophages [unpublished data, 2005]), occlusive coronary arterial atherosclerosis (cellular and acellular plaques containing lipid [including cholesterol clefts], collagen, and fibrin deposits3), and acute CHD when young (4 to 6 weeks old).2–4 At 6 weeks of age, dKO hearts exhibit multiple, large infarctions with extensive fibrosis around the ventricular outflow tract and patchy MIs in the apex, right ventricular wall and interventricular septum.5 In addition, they are hypertrophic with LV dilation, and exhibit severe dysfunction, including multiple electrocardiographic (ECG) abnormalities (ST elevation and depression, aneuysm formation, ventricular arrhythmias, AV blocks)), a 70% reduction in ±dP/dT, and 50% reduced ejection fraction. The dKO mice die between 5 and 8 weeks of age (mean 6 weeks).2,3 Similarities between dKO and human CHD raised the possibility that these mice may help to study the pathophysiology of CHD and to develop genetic, pharmacological, and environmental approaches for prevention and treatment.

Hepatic lipase (HL) hydrolyzes triglycerides and phospholipids and is involved in processing chylomicron remnants, intermediate-density lipoprotein and HDL.5 HL is primarily synthesized and secreted by the liver and is found in steroiogenic tissues.6 HL participates in conversion of intermediate-density lipoprotein to LDL and large lipid-rich HDL to smaller HDL, thereby modulating their relative plasma distributions.7–11 In addition to lipolytic activities, HL has ligand-binding activity and may mediate interactions of lipoproteins with cell surface proteoglycans and receptors, such as SR-B1 and LDL receptor-related protein, thus facilitating endocytosis and/or selective lipoprotein lipid uptake.12–16

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Many studies have demonstrated that hepatic lipase can influence atherosclerosis, although mechanisms through which this occurs are poorly understood. Increased HL activity has been linked to formation of small, dense proatherogenic LDL particles in humans. Conversely, reduced HL activity increases plasma HDL cholesterol levels in both humans (congenital deficiencies) and rodents (anti-HL antibodies or HL KO mice). ApoE/HL double KO mice exhibit significantly smaller aortic root atherosclerotic lesions than apoE single KO mice, despite an increase in plasma total and very-low-density lipoprotein cholesterol and altered lipoprotein structure and composition. There are also studies showing that HL can be anti-atherogenic. Thus, whereas HL modulates atherosclerosis, its precise effects vary depending on the system under study.

Here, we examined the role of atherosclerosis in CHD in dKO mice by examining the effects of HL deficiency. We found that HL deficiency significantly reduced aortic root and occulsive coronary arterial atherosclerosis, delayed the onset and/or progression of CHD, and increased longevity (37%). Our results support the proposal that occulsive coronary atherosclerosis is the likely cause of MI, cardiac dysfunction, and premature death in dKO mice. In addition, our study demonstrates that SR-BI is not essential for HL’s influence on atherogenesis.

**Methods**

SR-BI(--/--)apoE(--/--)HL(--/--) triple knockout (tKO) mice and control SR-BI(--/--)apoE(--/--)HL(+/+) dKO mice were generated on a 75:25 C57BL/6:SV129 background by crossing SR-BI(+/-)apoE(+/-) females with SR-BI(--/--)apoE(+/+) males. The offspring SR-BI(+/-)apoE(+/+)HL(+/+) females were then crossed to sibling SR-BI(--/--)apoE(+/+)HL(+/+) males to generate littermate tKO and dKO mice as well as breeder mice that were used to maintain the colonies. Animals were fed standard chow (Prolab 3000; PMI Feeds, St. Louis, Mo) and housed as previously described and experiments followed MIT Animal Care guidelines. No significant differences were observed between males and females. Genotypes were determined by polymerase chain reaction (PCR).

All morphological, histochemical, biochemical, and cardiac functional analyses were performed as described previously or in the online data supplement (see http://atvb.ahajournals.org).

**Results**

To assess the effects of inactivation of the HL gene on CHD in SR-BI/apoE dKO mice, we generated from common precursor animals 2 new strains on genetically similar mixed 75:25 C57BL/6:SV129 backgrounds: SR-BI/apoE HL tKO mice and control SR-BI/apoE dKO mice. The effect of HL gene inactivation on lifespan of dKOs is shown in Figure 1 (dKO, black curve, tKO, gray). The tKOs survived 37% longer than dKOs (mean lifespans (days): dKO, 46.0±0.3 (6.6 weeks, n=160); tKO, 63.0±0.8 (9 weeks, n=94) (P<0.0001 Logrank test). We also compared the lifespans of sibling dKOs and tKOs generated by intercrossing SR-BI(+/--)/apoE(+/--)/HL(+/--) mice. These tKO mice lived 12 to 37 days (25% to 84%) longer than their dKO siblings [n(tKO)=15, n(dKO)=16]. In both strains, a small fraction of mice (dKOs, 4.8%; tKOs, 10.5%) died within 10 days of weaning, possibly because of hypersensitivity to the anesthetic used during sampling for genotyping or as yet unidentified relatively uncommon genetic or environmental factors. These animals are included in Figure 1, but not in the calculation of mean lifespans.

At 6 weeks of age, dKOs appeared hunched and sickly (eg, lethargy) with ruffled fur, whereas tKOs were healthier (sleeker fur, more active). Moreover, at this age tKOs weighed significantly more than dKOs (18.5±0.4 versus 16.4±0.3 gm). By 9 weeks the tKOs had grown larger (20.9±0.7 gm), but otherwise resemble the ill 6-week-old dKOs. To investigate mechanisms underlying the extended lifespan of the tKOs, we further characterized the mice at ≈6 weeks (37 to 48 days) of age, designated dKO-6 and tKO-6 and ≈9 weeks (60 to 68 days, tKO-9). As previously reported, by 6 weeks of age ≈50% of the dKO mice died and the surviving animals exhibited occulsive atherosclerosis, MI, and heart dysfunction, whereas virtually all of the tKO mice were alive. We expected that surviving tKO-9 mice (mean age of death) might resemble dKO-6 mice.

**Cardiac Function and Structure**

As previously reported, dKO-6s exhibited a variety of electrocardiographic (ECG) abnormalities associated with CHD, including ST depression and ST elevation indicative of ischemia and myocardial infarction (Figure 2A). In contrast, the ECGs of tKO-6s were normal (Figure 2B). However, as the tKOs approached 9 weeks of age, they exhibited ECG abnormalities similar to those of dKO-6s (eg, ST depression and elevation; Figure 2C), suggesting that, as is the case for dKOs, CHD may be the primary cause of premature death. Thus, inactivation of HL may have slowed the initiation and/or progression of CHD. The gross characteristics of the hearts support this suggestion. Hearts from tKO-6 mice were similar in surface appearance to CHD-free control hearts (Figure 3A and 3C), whereas those from dKO-6s and tKO-9s were markedly enlarged and exhibited surface patches characteristic of large MIs (Figure 3B and 3D). Although the heart-to-body weight ratio for tKO-6s was significantly larger than that for controls (1.3-fold), indicating that the tKO-6 hearts were not normal; this cardiomegaly, caused at least in part by hypertrophy, was significantly less than that of dKO-6s (1.9-fold) or tKO-9s (1.7-fold) (Figure 3E).
Masson’s trichrome stained longitudinal heart sections (Figure 4A to 4C, healthy myocardium stains red, fibrotic tissue blue) showed that, unlike the massive fibrosis/MI present in virtually all dKO-6 and tKO-9 hearts (Figures 4A and 4C), with especially marked left ventricular dilation and fibrosis in the outflow tracts of the most ill tKO-9s, tKO-6 hearts were relatively healthy, with little fibrosis (Figure 4B) or intramyocardial neutral lipid deposition (Oil Red O staining, data not shown). Thus, tKO mice develop MIs, cardiomegaly, left ventricular dilation, and cardiac dysfunction (ECGs) similar to those of dKO mice, but onset and/or progression of disease is slower in tKOs.

Effects of Hepatic Lipase Deficiency on Aortic Root and Occlusive Coronary Arterial Atherosclerosis

To determine whether extended lifespan and improved cardiac pathology of HL-deficient tKO-6 mice was associated with reduced atherosclerosis, we quantified aortic root and occlusive coronary arterial atherosclerosis. Figure 4 shows representative images of Oil Red O-stained aortic roots (Figure 4G to 4I) and quantitative analysis of lesion sizes (Figure 4J). Compared with lesion areas in dKO-6s, the plaques were 3-fold smaller in tKO-6 (Figure 4H) and 2.5-fold larger in tKO-9 (Figure 4I) mice.

Comparable results were observed in coronary arteries. Vessels were scored in at least 5 Oil Red O-stained sections per mouse and divided into 3 categories: severely (>50%) occluded, partially (<50%) occluded, and open (no visible plaque). In dKO-6 mice (Figure 4D and 4K), severe and partially occluded lesions were common (36%±4%, 28%±3%, respectively, n=5 mice), whereas 36%±3% of the vessels were open. Occlusions, which were either predominantly acellular or contained significant cellular components, were prevalent in areas with myocardial fibrosis, especially near the upper ventricular outflow regions. Unlike the dKO-6s, most vessels in tKOs-6s (Figure 4E and 4K), values comparable to those in dKO-6s. The extents of aortic root and occlusive coronary arterial atherosclerosis were grossly correlated with the extent of MI, cardiomegaly, cardiac function, and lifespan, suggesting atherosclerosis is most likely responsible for CHD and premature death in dKO mice and that HL-deficiency may extend life by slowing the onset and/or progression of atherogenesis. For tKO-9 and dKO-6 mice, there was a substantially larger difference in aortic root atherosclerosis (≈3-fold) than in the number of occluded coronary vessels (1.02-fold), even though sampling occurred at their respective mean ages of death (9 or 6 weeks). Occlusive coronary arterial atherosclerosis is expected to cause myocardial ischemia and consequently MI, cardiac dysfunction, and death, whereas previous studies have shown that extensive aortic root atherosclerosis alone is not usually associated with MI and overt cardiac dysfunction. Thus, the closer correlation of occlusive arterial than aortic root atherosclerosis with the cardiac phenotypes is not surprising.

Effects of Hepatic Lipase Deficiency on Lipoprotein Abundance and Composition

Figure 5 and the Table show that HL deficiency had little influence on the size distribution of the lipoproteins in dKOs and only a small effect on relative lipid compositions. At 6 weeks of age the fasting plasma total cholesterol level in tKOs was ≈1.6-fold higher than in dKOs, a difference caused primarily by increases in the very-low-density lipoprotein size range (Figure 5); however, by 9 weeks of age the plasma total cholesterol in tKOs decreased to that in dKO-6s. Nonfasting (Table I, available online at http://atvb.ahajournals.org) and fasting plasma lipid levels were similar. Based on comparisons with healthy SR-BI+/−/apoE−/−/HL−/− littermates of tKO mice at 6 (n=5, TC=745±58) and 9 (n=7, TC=817±90) weeks of age, the decrease in plasma cholesterol between 6 and 9 weeks in tKO mice was
not simply attributable to normal aging and/or sexual maturation. The mechanisms underlying this reduction are unclear, although it is noteworthy that higher lipoprotein levels may be cardioprotective, possibly by limiting inflammation, and reduced lipid levels may be associated with a poor prognosis in chronic heart failure.27,28

We observed no significant differences in the fasting triglyceride levels of tKO-6 and dKO-6 mice or the HDL cholesterol levels of dKO-6, tKO-6, and tKO-9 mice (Table 19,20). Though triglyceride levels were similar in dKO-6 and tKO-6 mice, they were higher in tKO-9 mice. The ratios of surface (phospholipids plus unesterified cholesterol) to core (cholesterol ester plus triglyceride) lipids were similar for dKO-6s and tKO-9s and significantly higher than in tKO-6s (Table). As previously reported, higher lipoprotein surface to core lipid ratios are associated with greater atherogenic potential in different SR-BI–deficient models of CHD.4,23 Thus, we did not observe substantial HL-dependent differences in the lipoproteins. Further studies are necessary to determine whether subtle differences in lipoprotein structures caused by HL deficiency, or if HL-dependent changes in lipoprotein metabolism distinct from changes in particle structure (eg, as a consequence of the lipoprotein binding activity of HL),29 were responsible for the striking effects on atherosclerosis in dKO mice.

Discussion
The SR-BI/apoE dKO mouse exhibits many hallmarks of human CHD (see Introduction).3 It and its variant, the SR-BI KO/ApoE6/10b mouse,23 provide uniquely powerful tools to investigate the mechanisms underlying CHD and to identify potential therapeutic targets and approaches. For their promise to be realized, it is necessary to determine whether the CHD etiology and pathology in these models are similar to those in humans. After initial characterization of dKO mice,2,3 there appeared to be two potential causes of MIs, cardiac dysfunction, and premature death: B- and T-cell–mediated inflammatory heart disease or ischemic disease caused by occlusive atherosclerosis. Previous studies, in which B- and T-lymphocyte production was blocked by deficiency in the RAG2 gene, demonstrated that B- and
T-lymphocyte–mediated inflammatory heart disease is not essential for CHD in dKO mice.21

In an attempt to directly test the role of atherosclerosis in this CHD, we generated SR-BI/apoE/HL tKO mice with the goal of changing the rate of onset or progression of atherosclerosis. Numerous reports indicate that HL can have either pro-atherogenic or anti-atherogenic activities in humans and mice, depending on the details of the system undergoing study.30,31,32 For example, alterations in HL expression result in strikingly different effects in apoE KO and LDLR KO mice, despite the similarities in morphology of their aortic atherosclerotic lesions. HL deficiency dramatically reduces aortic root atherosclerosis in apoE KO mice33 via poorly understood mechanisms that likely include alterations in lipoprotein metabolism, indicating a pro-atherogenic role for HL. In contrast, hepatic overexpression of human HL in LDLR/HL double KO mice reduces atherosclerosis, indicating an anti-atherogenic activity of overexpression.33

Unlike the dKO mice examined here, these and other murine models do not exhibit significant occlusive coronary arterial atherosclerosis. Thus, the influence of HL on murine atherosclerosis of this sort has not been defined previously; however, examination of HL effects on coronary arterial atherosclerosis in humans has been reported.30

The diverse effects of HL on atherosclerosis in different systems is not surprising, because HL is a complex protein that exhibits distinct lipolytic16 and ligand-binding13–15,38–41 activities that have multiple effects on lipoprotein composition and metabolism, and thus could influence atherogenesis via a variety of mechanisms.9,42 For example, in apoE KO mice, HL deficiency may be anti-atherogenic because it raises HDL cholesterol, increases the capacity of HDL to promote cellular cholesterol efflux in vitro,19 prevents HL-mediated increases in the atherogenicity of very-low-density lipoprotein particles,19,34,35 increases plasma apoA-I and apoA-IV levels,19,20 or alters the phospholipid composition of lipoproteins.20,36,37 In addition, expression of catalytically inactive HL has been shown to produce a dramatic reduction of atherosclerosis in apoE/HL double KO mice,29 but not in LDLR/HL double KO mice.33 Furthermore, HL might influence atherosclerosis independently of its effects on systemic lipoprotein metabolism. For example, bone marrow transplantation experiments demonstrate localized HL expression by macrophages can dramatically influence aortic atherosclerosis.9,42 Thus, the effects of HL deficiency on aortic root, and particularly on coronary arterial, atherosclerosis in dKO mice could not be known a priori.

HL deficiency significantly reduced both aortic root and coronary arterial occlusive atherosclerosis in dKO mice, with 64% and 42% reductions, respectively, at 6 weeks of age. Despite the striking effects of HL deficiency on atherosclerosis, we did not observe alterations in relative cholesterol and phospholipid levels in lipoproteins or the size distribution of lipoproteins, although as expected HL deficiency was accompanied by increased plasma total cholesterol. Thus, as in other model systems, the precise mechanisms through which HL deficiency reduces atherosclerosis in dKO mice remain unclear. Future studies will be required to explore the possibility that in dKO mice HL deficiency resulted in subtle, yet functionally important, changes in lipoprotein structure, binding to tissues, or metabolism.

As a consequence we could examine the effects of the reduced atherosclerosis on CHD. At 6 weeks of age, the reduced atherosclerosis in tKO mice was associated with a dramatic reduction in hypertrophy and almost complete prevention of MI, and electrocardiographically determined cardiac dysfunction (eg, ST elevation and depression). Furthermore, in tKO mice there was an increase in the mean age of death from ≈6 to 9 weeks. By 9 weeks of age, occlusive coronary arterial atherosclerosis in tKO mice was virtually identical to that in 6-week-old dKO mice (65% versus 64% of arteries with occlusions) and there was 1.5-fold greater aortic root disease. The abnormal cardiac phenotypes in 9-week-old tKO mice resembled those of 6-week-old dKOs. Thus, the extent of occlusive coronary arterial atherosclerosis appeared to be somewhat more closely correlated with cardiac pathology than the relative amounts of aortic root atherosclerosis.

The current study demonstrates that HL deficiency can delay the onset and/or reduce the rate of progression of atherosclerosis in the absence of SR-BI, and it seems likely that this reduction in occlusive coronary arterial atherosclerosis was responsible for the slower development of CHD and the extension of lifespan. Two other studies support this conclusion. First, the hypolipidemia and anti-atherosclerosis

### Table 1. Fasting Plasma Lipid Data From dKO-6, tKO-6, and tKO-9 Mice

<table>
<thead>
<tr>
<th></th>
<th>dKO-6 n=32†</th>
<th>tKO-6 n=20†</th>
<th>tKO-9 n=12†</th>
<th>P (dKO-6 vs tKO-6)‡</th>
<th>P (dKO-6 vs tKO-9)‡</th>
<th>P (tKO-6 vs tKO-9)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (TO)*</td>
<td>895±33</td>
<td>1419±73</td>
<td>906±59</td>
<td>0.0001</td>
<td>0.8579</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Unesterified cholesterol (UC)*</td>
<td>694±22</td>
<td>997±43</td>
<td>710±38</td>
<td>0.0001</td>
<td>0.7187</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Phospholipids (PL)*</td>
<td>545±18</td>
<td>838±38</td>
<td>602±29</td>
<td>0.0001</td>
<td>0.1110</td>
<td>0.0001</td>
</tr>
<tr>
<td>Triglycerides*</td>
<td>55±5 (31)</td>
<td>47±6 (19)</td>
<td>82±9</td>
<td>0.2894</td>
<td>0.0055</td>
<td>0.0025</td>
</tr>
<tr>
<td>HDL cholesterol*</td>
<td>219±7 (28)</td>
<td>229±6 (19)</td>
<td>220±11 (11)</td>
<td>0.3016</td>
<td>0.9144</td>
<td>0.4373</td>
</tr>
<tr>
<td>Surface-to-core ratio§</td>
<td>5.2±0.3 (31)</td>
<td>4.2±0.2 (19)</td>
<td>5.1±0.4</td>
<td>0.0067</td>
<td>0.9013</td>
<td>0.0026</td>
</tr>
<tr>
<td>UC/TC ratio</td>
<td>0.78±0.01</td>
<td>0.71±0.01</td>
<td>0.79±0.02</td>
<td>0.0001</td>
<td>0.5810</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PL/TC ratio</td>
<td>0.61±0.01</td>
<td>0.60±0.01</td>
<td>0.67±0.02</td>
<td>0.2951</td>
<td>0.0048</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

*mg/dL
†n=number of animals per group (variations in parentheses).
‡P values for comparisons determined using unpaired Student t test, 2-tailed.
§Surface to core lipids=(PL+UC)/(esterified cholesterol+TG), where esterified cholesterol=TC–UC.23

References

1. [Technical reference](http://atvb.ahajournals.org/). Downloaded on April 5, 2017.
drug probucol dramatically blocks in dKO mice the onset and/or progression of atherosclerosis and cardiac pathology, and increases their lifespans (mean age of death increases to 36 weeks). However, it is difficult to draw definitive conclusions about the role of lipoprotein metabolism and atherosclerosis in the CHD in dKO mice solely from the beneficial effects of probucol, because this drug exhibits multiple, pleiotropic activities (anti-oxidant, anti-inflammatory, cardioprotective in the absence of dyslipidemia). Second, a lipid-rich atherogenic diet can induce in SR-BI KO/ApoR6 lth mice fatal CHD that is remarkably similar to that in chow-fed dKO mice. SR-BI KO/ApoR6 lth mice have low, rather than no, plasma apoE, rendering them susceptible to diet-induced hyperlipidemia, atherosclerosis and CHD.

Taken together with our earlier studies, this work supports the suggestion that occlusive coronary atherosclerosis is directly responsible for ischemia-induced myocardial infarction, which in turn leads to cardiac dysfunction and premature death, a pathologic process closely resembling that in human CHD. Because rapid onset occlusive coronary arterial atherosclerosis, (to date, occlusive thrombi have not been observed in these mice [unpublished data, 2005]), associated MI does not usually accompany the aortic atherosclerosis seen in other common murine models of hyperlipidemia, provide attractive small animal models of human occlusive atherosclerotic CHD for genetic and pharmacological studies of the mechanisms underlying the most common causes of heart disease and preclinical testing of new therapeutic strategies.

Acknowledgments

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SUPPLEMENTAL MATERIAL

METHODS

Mice: Animals were housed in micro-isolater cages and fed a standard chow diet. Experiments followed MIT and NIH Animal Care guidelines. Experiments other than survival studies were conducted on dKOs age 37 to 48 days and tKOs aged 37 to 68 days. No significant differences were observed between males and females. Genotypes were determined by PCR as previously described$^1,^2$. HL-/- mice were obtained from Jackson Laboratory (Bar Harbor, ME). SR-BI(-/-)/HL(-/-) mice were first generated by crossing C57BL/6 HL(-/-) mice and 50:50 C57BL/6:SV129 SR-BI (-/-) mice and then intercrossing their offspring. SR-BI (-/-)/apoE (-/-)/HL (-/-) triple knockout (tKO) mice and control SR-BI (-/-)/apoE (-/-)/HL (+/+), double knockout (dKO) mice were generated on a 75:25 C57BL/6:SV129 background by crossing SR-BI(+/-)/apoE(-/-) females$^1$ with SR-BI(-/-)/ HL(-/-) males. The offspring SR-BI(+/-)/apoE(+/-)/HL(+/-) females were then crossed to sibling SR-BI(-/-)/apoE(+/-)/HL(+/-) males to generate littermate isolates of tKO and dKO mice as well as breeder mice that were used to maintain the colonies and generate subsequent experimental animals. HL genotype was determined via PCR using the primers 5’ TTC TCG GAG CAA AGT TCA CCT AAT 3’ and 5’ GTG ATT CTT CCA ATC TTG TTC 3’.

Plasma Lipid Composition and FPLC Lipoprotein Total Cholesterol Profiles: Plasma from nonfasted and 4 hour fasted animals was obtained from blood drawn at sacrifice by centrifugation at 14,000 rpm (Spectrafuge 16M) for 10 minutes at 4°C. Lipid concentrations were determined by enzymatic assays on plasma diluted 1:5 in phosphate buffered saline (PBS)
using kits (Cholesterol C-II, Free Cholesterol E and Phospholipids B) from Wako Chemical USA Inc., (Richmond, Virginia, USA) and the EZ HDL kit from Trinity Biotech USA (Berkeley Heights, NJ) ³. Plasma from nonfasted mice was diluted 1:4 in elution buffer (154 mM NaCl, 1 mM EDTA, pH 8) and subjected to FPLC analysis (total cholesterol determined for each fraction) either immediately following collection or after storage at 4°C as previously described². No significant differences in lipids were observed between males and females.

**Morphologic and Histologic Analyses:** Mice were weighed and anesthetized/euthanized with an overdose of 2.5% Avertin. Blood was drawn from the retro-orbital plexus with a heparinized capillary tube and used for hematocrits.

**Hematocrits:** Hematocrits were measured using microcapillary tubes and a hematocrit centrifuge. Hematocrits of dKO mice aged 41–48 days, tKO mice aged 40-46 days and tKO mice aged 61-67 days did not differ significantly from each other (31.6±0.7 [n=26], 32.6±1.5 [n=10] and 31.5±1.3 [n=10] respectively, P ANOVA=0.7555) but all differed significantly from control values (45.4 [n=13], P<0.0001). The effects of the dKO genotype on hematocrit has been reported elsewhere⁴.

**Gravimetry:** Intact hearts were collected from euthanized mice and rinsed clean of blood with heparin/PBS (10 units/ml) (heparin sodium salt, Sigma). Whole hearts and spleens were then blotted dry and weighed using an analytical balance.

**Histology:** Hearts were then immersed in cold Krebs-Hanseleit buffer (120 mM NaCl/25 mM NaHCO₃/3.3 mM KH₂PO₄/0.8 mM K₂HPO₄/1.2 mM MgCl₂/1.2 mM CaCl₂/10 mM glucose, pH 7.4) for 30 minutes, embedded in Tissue-TeK OCT compound (Sakura Finetek) and fresh frozen using dry ice/isopentane. Serial cryosections (10µm) cut onto Fisher MicroProbe Plus
slides (Fisher Scientific) were stained with Masson’s Trichrome (Sigma)\(^5\) or Oil red O and hematoxylin \(^1\).

**Quantification of Aortic Atherosclerosis:** Mice were sacrificed as described above. Hearts were perfused with cold PBS containing either 5mM EDTA or 10U/ml heparin, collected, immersed in Kreb-Hanseleit buffer and embedded in OCT\(^1,2\). Serial 10 \(\mu\)M sections were cut from the root of the aorta through aortic sinuses going up 350-400 \(\mu\)M (3-4 sections per slide). Sections were stained with Oil Red O and hematoxylin and eosin\(^1\). SPOT imaging software (Diagnostic Instruments, MI) was used to quantify the lesion area per cross section in ten to twenty sections per mouse which were then averaged to provide mean lesion area per mouse.

**Quantification of Occlusive Coronary Arterial Atherosclerosis:** Coronary occlusive atherosclerosis was quantified by counting vessels in five or more Oil Red O stained heart sections per mouse, and scoring vessels by visual inspection as open (essentially no atherosclerosis), partially (<50%) occluded or severely (>50%) occluded, and calculating the average percent of vessels in each category.

**ECGS:** ECGS were recorded and analyzed at MIT\(^6\).

**Statistical Analysis:** \(P \leq 0.05\) was considered significant (2-tailed, unpaired student’s \(t\) test or ANOVA test, GraphPad Prism 4.0). Survival curves employed the Kaplan-Meier function with the logrank test (GraphPad Prism 4.0). Values are expressed as mean \(\pm\) SEM.

**References**


Table 1: Nonfasting whole plasma lipid data for dKO-6, tKO-6 and tKO-9 mice.

<table>
<thead>
<tr>
<th>(mg/dl)</th>
<th>dKO (6 weeks)</th>
<th>tKO (6 weeks)</th>
<th>tKO (9 weeks)</th>
<th>P (dKO-6 vs. tKO-6)</th>
<th>P (dKO-6 vs. tKO-9)</th>
<th>P (tKO-6 vs. tKO-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=18</td>
<td>n=15</td>
<td>n=9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (TC)</td>
<td>835±32</td>
<td>1395±66</td>
<td>963±67</td>
<td>&lt;0.0001</td>
<td>0.0603</td>
<td>0.0003</td>
</tr>
<tr>
<td>Unesterified Cholesterol (UC)</td>
<td>618±25</td>
<td>1041±47</td>
<td>746±58</td>
<td>&lt;0.0001</td>
<td>0.0254</td>
<td>0.0008</td>
</tr>
<tr>
<td>Phospholipids (PL)</td>
<td>546±26</td>
<td>947±37</td>
<td>673±53</td>
<td>&lt;0.0001</td>
<td>0.0213</td>
<td>0.0003</td>
</tr>
<tr>
<td>UC/TC Ratio</td>
<td>0.74±0.01</td>
<td>0.75±0.01</td>
<td>0.77±0.01</td>
<td>0.5840</td>
<td>0.0840</td>
<td>0.2308</td>
</tr>
<tr>
<td>PL/TC Ratio</td>
<td>0.65±0.01</td>
<td>0.68±0.01</td>
<td>0.70±0.02</td>
<td>0.0785</td>
<td>0.0375</td>
<td>0.5685</td>
</tr>
</tbody>
</table>

‘n’ = number of animals per group with deviations indicated in parentheses. P-values for pairwise comparisons were determined using unpaired student’s t-test, 2 tailed.