Cytochrome P450 1B1 Contributes to the Development of Atherosclerosis and Hypertension in Apolipoprotein E–Deficient Mice

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Abstract—Cytochrome P450 (CYP) 1B1 contributes to vascular smooth muscle cell growth and hypertension in male mice. This study was conducted to determine the contribution of CYP1B1 to the development of atherosclerosis and hypertension and associated pathogenesis in 8-week-old male apolipoprotein E–deficient (ApoE−/−/Cyp1b1−/−), and ApoE- and CYP1B1-deficient (ApoE+/−/Cyp1b1−/−) mice fed a normal or atherogenic diet for 12 weeks. A separate group of ApoE−/−/Cyp1b1−/− mice on an atherogenic diet was injected every third day with the CYP1B1 inhibitor, 2,3′,4,5′-tetramethoxystilbene (300 μg/kg), or its vehicle, dimethyl sulfoxide (30 μL, IP); systolic blood pressure was measured by the tail cuff method. After 12 weeks, mice were euthanized, blood collected for lipid analysis, and aortas harvested for measuring lesions and remodeling, and for infiltration of inflammatory cells by histological and immunohistochemical analysis, respectively, and for reactive oxygen species production. Blood pressure, areas of lipids and collagen deposition, elastin breaks, infiltration of macrophages and T lymphocytes, reactive oxygen species generation in the aorta, and plasma lipid levels were increased in ApoE−/−/Cyp1b1−/− mice on an atherogenic diet; these changes were minimized in mice given 2,3′,4,5′-tetramethoxystilbene, and in ApoE+/−/Cyp1b1−/− mice on an atherogenic diet; absorption/production of lipids remained unaltered in these mice. These data suggest that aortic lesions, hypertension, and associated pathogenesis in ApoE−/−/Cyp1b1−/− mice on an atherogenic diet are most likely dependent on CYP1B1-generated oxidative stress and increased plasma lipid levels independent of blood pressure and absorption of lipids. CYP1B1 could serve as a novel target for developing drugs to treat atherosclerosis and hypertension caused by hypercholesterolemia. (Hypertension. 2016;67:206-213. DOI: 10.1161/HYPERTENSIONAHA.115.06427.) ● Online Data Supplement

Key Words: apolipoprotein E, deficiency ■ cytochrome P450 1B1 ■ diet, atherogenic ■ plasma lipids ■ vascular, remodeling

Cytochrome P450 (CYP) 1B1, a member of the CYP enzyme family I, subfamily B, polypeptide 1, that was cloned in 1994 is expressed in several nonhepatic tissues including the cardiovascular system. In blood vessels, CYP1B1 is expressed primarily in vascular smooth muscle cells (VSMCs) with very low expression in endothelial cells, and it is increased by shear stress. However, CYP1B1 is also expressed in retinal endothelial cells, where it is involved in angiogenesis in response to hypoxia. CYP1B1 is constitutively active and can metabolize several substrates including fatty acids, steroids, and retinoids. CYP1B1 also metabolizes procarcinogens, polycyclic aromatic hydrocarbons, that promote development of tumors and atherosclerosis and result in formation of DNA adducts in VSMCs. Previous studies from our laboratory have shown that CYP1B1 contributes to hypertension and associated pathogenesis, including activation of nicotinamide adenine dinucleotide phosphate oxidase and generation of reactive oxygen species (ROS), inflammation, and endothelial dysfunction in various experimental animal models. We have also shown that VSMC migration, proliferation, and hypertrophy caused by angiotensin II (Ang II) are mediated by CYP1B1-dependent production of ROS.

The increased ROS production that results in endothelial dysfunction is also observed in atherosclerosis, a chronic disease characterized by the accumulation of lipids in the arterial wall, leading to the formation of plaques that can cause heart attacks and strokes. Therefore, inhibiting CYP1B1 activity could be a potential strategy for treating atherosclerosis and its complications.

This study was conducted to determine the contribution of CYP1B1 to the development of atherosclerosis and hypertension and associated pathogenesis in 8-week-old male apolipoprotein E–deficient, ApoE−/−/Cyp1b1−/−, and ApoE- and CYP1B1-deficient, ApoE+/−/Cyp1b1−/− mice fed a normal or atherogenic diet for 12 weeks. A separate group of ApoE−/−/Cyp1b1−/− mice on an atherogenic diet was injected every third day with the CYP1B1 inhibitor, 2,3′,4,5′-tetramethoxystilbene (300 μg/kg), or its vehicle, dimethyl sulfoxide (30 μL, IP); systolic blood pressure was measured by the tail cuff method. After 12 weeks, mice were euthanized, blood collected for lipid analysis, and aortas harvested for measuring lesions and remodeling, and for infiltration of inflammatory cells by histological and immunohistochemical analysis, respectively, and for reactive oxygen species production. Blood pressure, areas of lipids and collagen deposition, elastin breaks, infiltration of macrophages and T lymphocytes, reactive oxygen species generation in the aorta, and plasma lipid levels were increased in ApoE−/−/Cyp1b1−/− mice on an atherogenic diet; these changes were minimized in mice given 2,3′,4,5′-tetramethoxystilbene, and in ApoE+/−/Cyp1b1−/− mice on an atherogenic diet; absorption/production of lipids remained unaltered in these mice. These data suggest that aortic lesions, hypertension, and associated pathogenesis in ApoE−/−/Cyp1b1−/− mice on an atherogenic diet are most likely dependent on CYP1B1-generated oxidative stress and increased plasma lipid levels independent of blood pressure and absorption of lipids. CYP1B1 could serve as a novel target for developing drugs to treat atherosclerosis and hypertension caused by hypercholesterolemia. (Hypertension. 2016;67:206-213. DOI: 10.1161/HYPERTENSIONAHA.115.06427.)
inflammatory disorder initiated by injury to the endothelium and associated with hypertension, diabetes mellitus, hyperlipidemia, and smoking. Endothelial cell damage promotes adhesion of monocytes, which migrate in the subendothelium where they become macrophages.\textsuperscript{16–18} ROS produced by macrophages and smooth muscle cells oxidize low-density lipoproteins (LDL) into oxidized LDL, which accumulates in macrophages and results in formation of specialized foam cells that give the appearance of yellow color fatty streaks or plaques.\textsuperscript{16–18} Cytokines produced by inflammatory, endothelial, and smooth muscle cells stimulate migration and proliferation of VSMCs that form fibrous caps covering fatty streaks.\textsuperscript{16–18} Because CYP1B1-dependent pathological events in animal models of hypertension (including ROS production, endothelial dysfunction, and inflammation\textsuperscript{11–14}) are also observed in atherosclerosis,\textsuperscript{16–18} it led us to hypothesize that atherosclerosis and associated pathogenesis caused by hyperlipidemia are mediated by a CYP1B1-dependent alteration in lipid levels and oxidative stress. To thoroughly test this hypothesis, we investigated the effect on the development of atherosclerotic aortic lesions (AAL) and associated pathogenesis including hypertension and the underlying mechanism of the selective CYP1B1 inhibitor 2,3,4,5-tetramethoxystilbene (TMS),\textsuperscript{19} and Cyp1b1 gene disruption in ApoE knockout mice (ApoE\textsuperscript{−/−}) fed a normal diet (ND) or atherogenic diet (AD). The results showed that CYP1B1 is essential for increased plasma lipid levels, development of AAL, vascular damage, and hypertension in ApoE\textsuperscript{−/−} mice fed AD, most likely by increased oxidative stress independent of lipid absorption.

**Methods**

All experiments were conducted according to protocols approved by our Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−}, ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{+/−}, ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{+/+}, and double knockout (ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−}) 8-week-old mice were fed ND or AD. Another group of ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice on AD were injected with CYP1B1 inhibitor, TMS (300 µg/kg, IP), or its vehicle (dimethyl sulfoxide, 30 µL) every third day. Aortas were isolated for histological and immunohistochemical characterization of atherosclerotic lesions. Plasma levels of lipids were measured. A separate series of experiments was performed to determine the absorption/production of lipids. Detailed experimental methods are available in the online-only Data Supplement.

**Statistical Analysis**

Data were analyzed by 1-way ANOVA, and the difference between groups was determined using Newman–Keul’s post hoc test for multiple comparisons or Student t test for comparison of 2 groups. The average values of different parameters from 3 to 5 different experiments were expressed as the mean±SEM. 

\[ P<0.05 \] was considered statistically significant.

**Results**

**CYP1B1 Inhibitor TMS or Cyp1b1 Gene Disruption Minimized Development of AAL in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} Mice on AD**

In ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice on AD for 12 weeks, en face analysis of longitudinally opened descending thoracic and abdominal aorta showed atherosclerotic lesions. These lesions were reduced in Cyp1b1 gene-disrupted and ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice treated with TMS, but not its vehicle, dimethyl sulfoxide (Figure 1A and 1B). AAL were also observed in ascending aortic sections from ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice fed AD but not mice fed ND and in mice treated with TMS. No lesions were found in ascending aortic sections of ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice fed ND or AD (Figure 1C). Because we did not observe any significant AAL in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} and ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice fed ND or AD, additional studies in these mice were not performed. AD diet did not alter aortic Cyp1b1 mRNA in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} or ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice (Figure S1 in the online-only Data Supplement).

**TMS Reduced CYP1B1 Activity Without Altering Its Protein Expression in the Heart**

The activity of CYP1B1 and its expression were measured in the heart because of limited amount of aorta available. Cardiac activity of CYP1B1 was increased by AD when compared with ND in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−}. Treatment of mice with TMS but not its vehicle dimethyl sulfoxide inhibited cardiac CYP1B1 activity. None of the treatments altered cardiac expression of CYP1B1 protein in these mice (Figure S2).

**TMS or Cyp1b1 Gene Disruption, Preserved Integrity of Extracellular Matrix in the Proximal Aorta of ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} Mice on AD**

To determine whether the CYP1B1 inhibitor TMS or Cyp1b1 gene disruption protects against the vascular pathogenesis associated with atherosclerosis in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice on AD, collagen deposition was examined by Masson Trichrome staining and elastin fiber structural integrity by staining with Verhoff Van Gieson in the proximal aorta. In ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice on AD, the total collagen content and number of elastin breaks in aorta were increased when compared with mice on ND. Treatment with TMS suppressed collagen content (Figure 2A and 2B) and prevented elastin breaks (Figure 3A and 3B). In ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice fed AD, aortic collagen content remained unaltered (Figure 2A and 2B), and breaks in elastin fibers were minimized (Figure 3A and 3B).

**TMS or Cyp1b1 Gene Disruption, Minimized Inflammatory Response in the Proximal Aorta of ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} Mice on AD**

Monocytes/macrophages and T lymphocytes play an important role in the development of inflammation and atherosclerosis.\textsuperscript{18} To assess the contribution of CYP1B1 to infiltration of monocyte/macrophages in AAL, we examined the effect of TMS and Cyp1b1 gene disruption on accumulation of F4/80\textsuperscript{+} macrophages and CD3\textsuperscript{+} T lymphocytes in AAL in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice fed AD. Increased infiltration of F4/80\textsuperscript{+} macrophages and CD3\textsuperscript{+} T lymphocytes was observed in the ascending aortic sections of ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} mice fed AD. Treatment with TMS and Cyp1b1 gene disruption minimized these changes (Figures S3 and S4, respectively).

**CYP1B1 Inhibitor TMS or Cyp1b1 Gene Disruption Decreases Aortic ROS Production and Plasma Levels of Thiobarbituric Acid Reactive Substances in ApoE\textsuperscript{−/−}/Cyp1b1\textsuperscript{−/−} Mice on AD**

ROS production, as indicated by an increase in 2-hydroxyethidium in aorta (Figure SSA and SSB), and plasma thiobarbituric acid reactive substances (Figure S5C), was increased in...
ApoE<sup>−/−</sup> mice fed AD; the increase was attenuated in ApoE<sup>−/−</sup>/Cyp1b1<sup>+/+</sup> mice treated with TMS and in ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice on AD (Figure S5A–S5C).

TMS or Cyp1b1 Gene Disruption Minimized the Increase in Systolic Blood Pressure and Endothelial Dysfunction in ApoE<sup>−/−</sup>/Cyp1b1<sup>+/+</sup> Mice on AD

Systolic blood pressure (SBP) increased significantly in ApoE<sup>−/−</sup>/Cyp1b1<sup>+/+</sup> mice during 12 weeks on AD when compared with mice fed ND, and treatment with TMS prevented this increase (Figure S6A). SBP was also minimized in ApoE<sup>−/−</sup>/Cyp1b1<sup>+/+</sup> mice fed AD when compared with ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice fed AD (Figure S6B). In ApoE<sup>−/−</sup>/Cyp1b1<sup>+/+</sup> and ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice, AD had no effect on endothelium-dependent or endothelium-independent dilation (Figure S7A and S7B). In ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> control mice fed AD, aortic endothelial function was impaired, as demonstrated by reduced dilation in response to acetylcholine; this impairment was prevented by TMS treatment (Figure S7C). Endothelium-independent dilation, measured by the relaxation of aorta in response to sodium nitroprusside, was not altered in ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice fed ND or AD with or without TMS (Figure S7D). No reduction in endothelium-dependent dilation was observed in ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice fed AD (Figure S7E);

endothelium-independent dilation was also unchanged in these mice (Figure S7F).

AAL Were Independent of Increase in SBP in ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> Mice on AD

To determine whether the AAL in ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice on AD were the result of increased SBP, these mice were placed on drinking water containing a direct vasodilator, hydralazine (125 mg/L), or its vehicle for 12 weeks. The mice were maintained on AD and SBP was measured every fourth week. In ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice on AD treated with vehicle, SBP was significantly increased and was associated with the development of AAL. However, in mice given hydralazine, the SBP increase was prevented, but these mice exhibited the same degree of AAL as did the vehicle-treated mice (Figure S8).

TMS or Cyp1b1 Gene Disruption Minimized the Increase in Plasma Lipid Levels in ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> Mice on AD

Eight-week-old ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice fed AD diet for 12 weeks had significantly increased plasma levels of cholesterol, triglycerides, LDL, and high-density lipoproteins compared with the mice fed ND. Treatment of ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> mice with TMS and Cyp1b1 gene disruption in these mice
(ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup>) fed AD significantly reduced the plasma levels of these lipids, except high-density lipoprotein (Table). The body weight in the different treatment groups measured at the 12th week before euthanizing the animals was not different (Table).

**TMS or Cyp1b1 Gene Disruption Did Not Alter Absorption/Production of Lipids in ApoE<sup>−/−</sup>/Cyp1b1<sup>+/−</sup> Mice on AD**

To determine whether the effect of TMS and Cyp1b1 gene disruption in decreasing plasma lipid levels in ApoE<sup>−/−</sup>/Cyp1b1<sup>+/−</sup>

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**Figure 2.** Cytochrome P450 (CYP) 1B1 inhibitor 2,3',4,5'-tetramethoxystilbene (TMS) or Cyp1b1 gene disruption minimizes collagen deposition in thoracic aorta of ApoE<sup>−/−</sup>/Cyp1b1<sup>+/−</sup> mice on atherogenic diet (AD). A, Collagen in aortic sections. B, Quantitation of total collagen in aortic sections. *P<0.05 ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD vs normal diet (ND), †P<0.05 ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD+TMS vs AD+dimethyl sulfoxide (DMSO), and ‡P<0.05 ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD vs. ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD (n=5 for each group of experiments; data are expressed as mean±SEM).

**Figure 3.** Cytochrome P450 (CYP) 1B1 inhibitor 2,3',4,5'-tetramethoxystilbene (TMS) or Cyp1b1 gene disruption abrogates elastin fiber breaks in the thoracic aorta of ApoE<sup>−/−</sup>/Cyp1b1<sup>+/−</sup> mice on atherogenic diet (AD). A, Elastin in thoracic aorta section. B, Quantitation of elastin fiber breaks in aortic sections. *P<0.05 ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD vs normal diet (ND), †P<0.05 ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD+TMS vs AD+dimethyl sulfoxide (DMSO), and ‡P<0.05 ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD vs ApoE<sup>−/−</sup>/Cyp1b1<sup>−/−</sup> AD (n=5 for each group of experiments; data are expressed as mean±SEM).
mice on AD was because of increase in uptake by the liver, decreased absorption, or production of lipids, we determined the liver triglyceride levels, fecal lipid contents, ratio of absorbable and nonabsorbable fat, and secretion of lipids from the liver and gut. Liver triglyceride levels were not altered in ApoE−/−/Cyp1b1+/+ mice fed AD when compared with those fed ND and remained unchanged by either treatment with TMS or in ApoE−/−/Cyp1b1−/− mice fed AD (Table). The food intake in ApoE−/−/Cyp1b1−/− and in ApoE−/−/Cyp1b1+/+ mice fed ND was higher than in those fed AD because of higher caloric content, but the total fecal lipid contents measured over a period of 72 hours were markedly increased in mice fed AD when compared with those fed ND. The fecal lipid contents were not altered in ApoE−/−/Cyp1b1−/− mice treated with TMS or in ApoE−/−/Cyp1b1−/− mice fed AD. None of these treatments altered body weight as measured in these mice over the same 72-hour period (Table S1).

The absorption of fat measured by the noninvasive method of determining the ratio of well-absorbed (coconut butter oil) and poorly absorbed fats (olestra; calcium soaps) containing the same 72-hour period (Table S1).

The secretion of lipids from the liver or gut or both as an indirect index of production/absorption of lipids was also determined by measuring plasma triglyceride levels after administering lipoprotein lipase and hepatic lipase inhibitor, proloxadorn 407 (P−407)20 with and without administering peanut oil by gavage in animals fasted for 12 hours (Methods in the Online Data Supplement). Two hours after administering P−407, the plasma levels of triglycerides were increased and remained elevated after 1 hour in ApoE−/−/Cyp1b1−/− mice. This effect of P−407 (increasing plasma triglyceride levels) was not altered in ApoE−/−/Cyp1b1−/− mice treated with TMS or in ApoE−/−/Cyp1b1−/− mice (Figure S9). Triglyceride levels increased 1 hour after administering peanut oil but returned to basal levels during the second hour in ApoE−/−/Cyp1b1−/− mice. In animals pretreated with P−407, administration of peanut oil to ApoE−/−/Cyp1b1−/− mice, markedly increased triglyceride levels after 2 hours that was significantly greater than with P−407 alone (Figure S9).

**Discussion**

This study demonstrated that CYP1B1 is critical for the development of AAL, associated inflammation, vascular damage, and hypertension in ApoE−/− mice fed AD, most likely by promoting increased oxidative stress and hyperlipidemia, which seems to be independent of lipid absorption. Eight-week-old ApoE−/−/Cyp1b1−/− mice fed AD but not ND for 12 weeks developed AAL in the descending thoracic and abdominal aorta and in sections of proximal aorta. Our finding, that treatment with TMS, an inhibitor of CYP1B1 activity,19 for 12 weeks in ApoE−/−/Cyp1b1−/− mice fed AD minimized AAL, suggests that CYP1B1 activity is required for the development of lesions. Although CYP1B1 is constitutively active, AD increased CYP1B1 activity without altering its expression as measured in the heart, whereas TMS inhibited its activity but not its expression. Because CYP1B1 is constitutively active, the increase in its activity by AD that could be because of biochemical modification of this enzyme remains to be determined. Further evidence that CYP1B1 is essential for the development of AAL was our finding that ApoE−/−/Cyp1b1−/− mice fed AD for 12 weeks did not exhibit atherosclerotic lesions in the descending and abdominal aorta.

Collagen accumulation and elastin disintegration resulting in breakdown of extracellular matrix are characteristic features of atherosclerosis.21,22 In ApoE−/−/Cyp1b1−/− mice, AD increased collagen deposition and breakdown of elastic fibers evaluated in aortic sections. Because these features were prevented by treatment with TMS in ApoE−/−/Cyp1b1−/− mice and in ApoE−/−/Cyp1b1−/− mice on AD, it seems that these effects of AD in ApoE−/−/Cyp1b1−/− mice depend on CYP1B1 activity. It is well established that atherosclerosis is an inflammatory disease and that macrophages and T lymphocytes contribute to the development of AAL.18 Our demonstration that increased infiltration of F4/80+ macrophages and CD3+ T cells in AAL caused by AD was attenuated by treatment with TMS in ApoE−/−/Cyp1b1−/− mice, and in ApoE−/−/Cyp1b1−/− mice fed

| Parameters Measured | ApoE−/−/Cyp1b1−/− Mice on AD | ApoE−/−/Cyp1b1−/− Mice on AD+DMSO | ApoE−/−/Cyp1b1−/− Mice on AD+TMS |
|---------------------|-------------------------------|-----------------------------------|----------------------------------|
| Body weight, g      | 26.7±0.7                      | 23.1±1.7                          | 22.6±1.7                         | 25.9±1.0                          | 27.3±1.1                          | 26.1±1.4                          |
| Plasma lipid profile, mmol/L |                      |                                   |                                  |                                  |                                  |                                  |
| Cholesterol         | 22.6±6.5                      | 85.4±6.8*                         | 77.1±9.2                         | 32.8±3.3†                         | 18.1±1.5                          | 25.8±4.6†                         |
| Triglycerides       | 8.8±4.2                       | 29.1±3.4*                         | 25.1±6.1                         | 7.1±1.1†                          | 6.4±1.2                           | 14.1±1.9‡                         |
| HDL                 | 7.4±1.6                       | 43.3±3.5*                         | 38.1±5.7                         | 25.7±1.8                          | 7.2±0.4                           | 24.9±2.2‡                         |
| LDL                 | 9.8±3.9                       | 36.3±3.9*                         | 33.1±4.9                         | 12.2±1.4†                         | 9.7±1.1                           | 22±3.2‡                           |
| Liver triglycerides (mg/g) | 76.3±10.6                    | 61.6±5.1                           | 73.9±3.3                         | 71.1±4.2                          | 62.7±11.7                         | 55.2±11.6                         |

Eight-week-old ApoE−/−/Cyp1b1−/− and ApoE−/−/Cyp1b1−/− mice were fed ND or AD for 12 weeks. AD indicates atherogenic diet; DMSO, dimethyl sulfoxide; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, normal diet; and TMS, 2,3,4,5′-tetramethoxy stilbene.

*P<0.05 ApoE−/−/Cyp1b1−/− AD vs ND.
†P<0.05 AD+DMSO vs AD+DMSO.
‡P<0.05 ApoE−/−/Cyp1b1−/− AD vs ApoE−/−/Cyp1b1−/− AD (n=4 for each group of experiments; data are expressed as mean±SEM).
AD suggests that CYP1B1 plays a crucial role in inflammation associated with AAL.

The mechanism by which AD in ApoE−/−/Cyp1b1+/+ mice produces AAL and associated vascular fibrosis, elastin fiber breakdown and inflammation could result from increased ROS production. ROS generated by nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenase, myeloperoxidase, and xanthine oxidase, and decreased NO has been implicated in the pathogenesis of atherosclerosis. 23 Deletion or inhibition of nicotinamide adenine dinucleotide phosphate oxidase, 24 12/15-lipoxygenase, 25 and myeloperoxidase 26 and xanthine oxidase 27 reduces, whereas long-term inhibition of NO synthesis 28 increases, atherosclerosis in animal models. ROS oxidizes LDL to oxidized LDL resulting in chemotaxis of monocytes. 29 ROS also activates immune cells and causes vascular inflammation. 30 Our demonstration that production of ROS in aorta and plasma levels of thiobarbituric acid reactive substances was increased in ApoE−/−/Cyp1b1+/+ mice and minimized in mice treated with TMS, and in ApoE−/−/Cyp1b1−− mice on AD suggests that ROS generated by CYP1B1 contributes to the pathogenesis of atherosclerosis. However, additional studies using high-performance liquid chromatograph are required to confirm the production of CYP1B1-dependent generation of ROS. CYP1B1 is expressed in extracellular tissues including VSMCs, 31,32 and macrophages 33 and can metabolize fatty acids, retinoic acid, and steroid hormones. 8,7,34 Our previous study in VSMCs showed that CYP1B1 metabolizes arachidonic acid and generates ROS, which by activating ≥1 signaling molecules, stimulates VSMCs migration and proliferation, and increases protein synthesis. 15 Our finding that an increase in thiobarbituric acid reactive substances levels in ApoE−/−/Cyp1b1+/+ mice on AD was inhibited by treatment with TMS or by Cyp1b1 gene disruption suggests generation of lipid peroxides by CYP1B1. Whether CYP1B1 also generates isoketals that form protein adducts in dendritic cells that activates immune cells, 34 contributing to the pathogenesis of AAL, remains to be determined. Lipid peroxidation products of arachidonic acid are elevated in carotid AAL of patients with symptomatic cerebrovascular disease. 35 Although there is substantial evidence supporting the role of oxidative stress in the development of AAL in animals, antioxidants have not been found in clinical studies in humans 36 to be effective against atherosclerosis. In view of the different sources of ROS and our paucity of our knowledge on the complex interaction among various oxidants and antioxidant enzyme systems and their substrates, additional studies are required to understand the limited protective effects of antioxidants against atherosclerosis in humans.

In the present study, AAL caused by AD in ApoE−/−/Cyp1b1+/+ mice was independent of body weight or SBP because, after 12 weeks, body weight in the different groups was not different. SBP was increased in ApoE−/−/Cyp1b1+/+ mice fed AD. However, the increase in SBP in ApoE−/−/Cyp1b1+/+ mice on AD was much higher than in those on ND and was associated with endothelial dysfunction as observed by diminished relaxation of aorta to acetylcholine but not to sodium nitroprusside. Our demonstration that, in ApoE−/−/Cyp1b1+/+ mice on AD, treatment with TMS or Cyp1b1 gene disruption (ApoE−/−/Cyp1b1−−) prevented the increase in SBP and endothelial dysfunction, suggests that CYP1B1 contributes to the rise in SBP and endothelial dysfunction in these mice. Previously, we reported that CYP1B1 contributes to Ang II–induced increase in BP and its associated pathogenesis including endothelial dysfunction in male mice by generating ROS and activating ≥1 signaling molecules. 37 Because Ang II promotes AAL in ApoE−/− mice 38 and angiotensin-converting enzyme or Ang II receptor blockers protect against atherosclerosis, 37,38 it is possible that Ang II via ROS production could contribute to increased SBP, endothelial dysfunction, and atherosclerosis in ApoE−/−/Cyp1b1+/+ mice on AD by a mechanism dependent on CYP1B1. The small increase in SBP produced in ApoE−/−/Cyp1b1−− mice on ND between 4 and 12 weeks that was not altered by either TMS or Cyp1b1 gene disruption is most likely age related. AAL produced by AD in ApoE−/−/Cyp1b1+/+ are unlikely the result of increased SBP because administration of hydralazine ≥12 weeks prevented increased SBP but not the development of atherosclerotic lesions in these mice. In ApoE−/−/Nos−− mice on AD or in 24-week-old ApoE−/− mice, hydralazine also decreased BP but did not prevent acceleration of AAL. 39 Because hydralazine stimulates renin release 40 and Ang II promotes atherosclerosis in ApoE−/− mice, 39 we cannot exclude the possibility that activation of the renin–angiotensin system masks the protective effect of hydralazine against AD-induced atherosclerosis. Moreover, whether hydralazine protects against collagen deposition, elastin break or inflammatory cell infiltration associated with AAL in ApoE−/−/Cyp1b1+/+ mice on AD remains to be investigated.

To determine whether TMS and Cyp1b1 gene disruption reduce AAL by altering lipid metabolism, we measured plasma lipid levels. Our finding that treatment with TMS or Cyp1b1 gene disruption minimized the increase in plasma levels of cholesterol, triglycerides, and LDL in ApoE−/−/Cyp1b1+/+ mice on AD could be because of decreased production or absorption of lipids. However, this scenario seems to be unlikely because treatment with TMS for 12 weeks or Cyp1b1 gene disruption in ApoE−/−/Cyp1b1+/+ mice on AD did not alter liver triglycerides levels. Also, the total fecal lipid contents measured over a 72-hour period in ApoE−/−/Cyp1b1+/+ and ApoE−/−/Cyp1b1−− mice on AD was increased when compared with those on ND; however, this increase was not altered in ApoE−/−/Cyp1b1+/+ mice pretreated with TMS for 2 weeks or in ApoE−/−/Cyp1b1−− mice. Moreover, TMS or Cyp1b1 gene disruption did not alter the ratio of absorbable and nonabsorbable fat in ApoE−/−/Cyp1b1−− mice measured by a noninvasive method. Furthermore, TMS or Cyp1b1 gene disruption did not seem to alter secretion of triglycerides from liver or gut, probably a reflection of production or absorption of fat, in ApoE−/−/Cyp1b1−− mice for the following reason. Administration of P-407, an inhibitor of lipoprotein and hepatic lipases 20 to these mice starved for 12 hours, markedly increased plasma triglyceride levels, and peanut oil given by gavage in mice pretreated with P-407 further increased plasma triglycerides. In both cases, treatment with TMS or Cyp1b1 gene disruption did not alter plasma triglycerides. However, additional
studies on the composition of lipids in lymphatic chylomicrons are required to evaluate the contribution of CYP1B1 to the process of lipid disposition. Moreover, the mechanism of the decrease in plasma lipid levels in \textit{ApoE}−/−/\textit{Cyp1b1}−/− mice by TMS and \textit{Cyp1b1} gene disruption that could result from altered lipoprotein, lipid metabolism, and bile salt production, or distribution of fat in adipose and other tissues and fatty acid oxidation remains to be determined. A recent study showed that \textit{Cyp1b1}-null mice exhibited altered lipid metabolism including resistance to high-fat diet-induced obesity and metabolic syndrome and reduced hyperlipidemia.\textsuperscript{44} However, the molecular mechanisms underlying these metabolic change remain to be elucidated. Moreover, the relationship between CYP1B1 and cyclooxygenase,\textsuperscript{45,46} lipoxygenase,\textsuperscript{28} and caveolin-1\textsuperscript{47,48} that have also been implicated in atherosclerosis needs to be explored.

In conclusion, this study provides evidence for the novel role of CYP1B1 in the development of atherosclerosis in male \textit{ApoE}−/−/\textit{Cyp1b1}−/− mice on AD and its pathogenesis including aortic accumulation of collagen, elastin fiber breaks, and infiltration of F4/80\textsuperscript{+} macrophages and CD3\textsuperscript{+} T cells, most likely by increased oxidative stress. Thus, the development of inhibitors of CYP1B1, like TMS, could be useful in treating atherosclerosis and hypertension caused by hypercholesterolemia.

**Perspectives**

The present study provides significant novel insight into the role of CYP1B1 in the development of atherosclerosis, hyperlipidemia, and associated pathogenesis including hypertension in male \textit{ApoE}−/−/\textit{Cyp1b1}−/− mice fed AD. Supporting this conclusion is our findings that a selective inhibitor of CYP1B1, TMS, and \textit{Cyp1b1} gene disruption in these mice minimizes aortic lesions and associated pathogenesis. These results suggest that CYP1B1 could be a target for treating atherosclerosis. However, it should be noted that CYP1B1 deficiency causes congenital glaucoma.\textsuperscript{46} This is likely because of a developmental abnormality in the eye as a result of no \textit{Cyp1b1} allele. A selective or chronic inhibition of CYP1B1 in adults may not cause increased ocular pressure. Therefore, this possibility needs to be evaluated experimentally for the viability of a CYP1B1 inhibitor in treating atherosclerosis.

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**Disclosures**

None.

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