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ORIGINAL RESEARCH

Testosterone Metabolite 6β-Hydroxytestosterone Contributes to Angiotensin II-Induced Abdominal Aortic Aneurysms in Apoe$^{-/-}$ Male Mice

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BACKGROUND: Sex is a prominent risk factor for abdominal aortic aneurysms (AAAs), and angiotensin II (Ang II) induces AAA formation to a greater degree in male than in female mice. We previously reported that cytochrome P450 1B1 contributes to the development of hypertension, as well as AAAs, in male mice. We also found that a cytochrome P450 1B1-generated metabolite of testosterone, 6β-hydroxytestosterone (6β-OHT), contributes to Ang II-induced hypertension and associated cardiovascular and renal pathogenesis in male mice. The current study was conducted to determine the contribution of 6β-OHT to Ang II-induced AAA development in *Apoe*$^{-/-}$ male mice.

METHODS AND RESULTS: Intact or castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ male mice were infused with Ang II or its vehicle for 28 days, and administered 6β-OHT every third day for the duration of the experiment. Abdominal aortas were then evaluated for development of AAAs. We observed a significant increase in the incidence and severity of AAAs in intact Ang II-infused *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, compared with vehicle-treated mice, which were minimized in castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice infused with Ang II. Treatment with 6β-OHT significantly restored the incidence and severity of AAAs in Ang II-infused castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. However, administration of testosterone failed to increase AAA incidence and severity in Ang II-infused intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice.

CONCLUSIONS: Our results indicate that the testosterone-cytochrome P450 1B1-generated metabolite 6β-OHT contributes to Ang II-induced AAA development in *Apoe*$^{-/-}$ male mice.

Key Words: 6β-hydroxytestosterone ■ abdominal aortic aneurysm ■ angiotensin II ■ castration ■ CYP1B1

Abdominal aortic aneurysm (AAA) is a chronic inflammatory vascular disease known to cause significant morbidity and mortality, especially among the aging population.[1–3] AAA is defined as a 50% increase in the diameter of the abdominal region of the aorta, compared with an adjacent arterial segment.[2] The disease progresses over the years or decades from a minor aortic dilation to eventual weakening of the vessel, thereby increasing its susceptibility to a deadly rupture. However, currently there are no pharmacological treatments available for AAAs.[4] It is well established that men have a higher risk of developing cardiovascular diseases, including hypertension and AAAs, than premenopausal women of the same age.[5,6] However, the underlying mechanisms for the propensity of men to develop AAAs are not fully understood. Similar to humans, in a mouse model of AAA development, angiotensin II (Ang II) infusion

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CLINICAL PERSPECTIVE

What Is New?

- This study demonstrated that 6β-hydroxytestosterone, a cytochrome P450 1B1-generated metabolite of testosterone, contributed to abdominal aortic aneurysm development and associated pathophysiological changes in *Apoe*$^{-/-}$ mice infused with angiotensin II.

What Are the Clinical Implications?

- Development of selective cytochrome P450 1B1 inhibitors could be useful to treat abdominal aortic aneurysms in men with high levels of angiotensin II, and those having testosterone supplements with cardiovascular disease.

Nonstandard Abbreviations and Acronyms

6β-OHT	6β-hydroxytestosterone
Ang II	angiotensin II
CYP1B1	cytochrome P450 1B1
ECM	extracellular matrix
MAD	maximal aortic diameter
SBP	systolic blood pressure
SMC	smooth muscle cell

was found to induce AAAs to a greater extent in male mice.[7]

Both sex hormones and sex chromosomes have been implicated in the development of AAA. Female mice with an XY sex chromosome complement develop severe Ang II-induced AAAs.[8] Although human studies with exogenous hormone treatments have yielded inconclusive results, the majority of studied animal models suggest a deleterious effect of male sex and androgens, with both a high incidence and increased size of AAAs in male *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice.[7,9] Moreover, castration of male mice resulted in a strikingly reduced incidence of Ang II-induced AAA formation compared with intact mice.[10] However, in castrated male rats, testosterone administration restored the increase in aortic diameter observed in an elastase infusion model of AAAs.[11]

Previously we reported that Ang II-induced hypertension and AAAs and their pathogenesis in male mice are dependent on cytochrome P450 (CYP) 1B1, a heme-thiolate monooxygenase expressed in cardiovascular tissues.[12,13] Also, we found that the testosterone-CYP1B1-derived metabolite 6β-hydroxytestosterone (6β-OHT) mediates Ang II-induced hypertension and cardiac and renal fibrosis in male mice.[14,15] These observations, together with our recent study showing that 6β-OHT contributes to vascular dysfunction and fibrosis in Ang II-induced hypertension in male mice,[16] led to the hypothesis that 6β-OHT mediates the effect of testosterone in the development of Ang II-induced AAA and its associated pathogenesis in male mice. To test this hypothesis, we investigated the effects of testosterone and 6β-OHT treatment on AAA incidence, severity, and pathogenesis, as well as systolic blood pressure (SBP) in *Apoe*$^{-/-}$, castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1* gene-disrupted (*Cyp1b1*$^{-/-}$) male mice. The results of our study showed that the testosterone-CYP1B1-derived metabolite 6β-OHT significantly contributed to the development of Ang II-induced AAAs and hypertension in male mice.

METHODS

The specific data, analytic methods, and study materials outlined below will be made available upon reasonable request to the corresponding author.

Materials

The following reagents were utilized for this study: Ang II from Bachem (Torrance, CA); 6β-OHT from Steraloids (Newport, RI); rat anti-CD68 and Dylight 549 goat anti-rat antibodies from Abcam (Cambridge, MA); dimethyl sulfoxide, hematoxylin and eosin, Masson's trichrome and elastin staining kits, α-actin antibody, and reagents for immunohistochemistry from Sigma-Aldrich (St. Louis, MO); Alzet osmotic pumps, model 2004 from Durect Corporation, (Cupertino, CA).

Animals

All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Tennessee and following the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME). *Apoe*$^{+/+}$/*Cyp1b1*$^{-/-}$ mice were generated at the National Cancer Institute, transferred to the University of Tennessee, backcrossed 10 generations to a C57BL/6J background, and then brother–sister mated to generate a homozygous line. *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice were generated in our laboratory at the University of Tennessee Health Science Center by breeding *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice with *Apoe*$^{+/+}$/*Cyp1b1*$^{-/-}$ mice from C57BL/6J backgrounds, as described previously.[13,17] Male *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice ≈16 weeks of age were used in all experiments.

Ang II-Induced AAAs in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ Mice

Mice were anesthetized with 1.5% isoflurane, and Alzet osmotic pumps were implanted subcutaneously to infuse 700 ng/kg per minute of Ang II or saline (as vehicle control) for 28 days. Animals of both genotypes (*Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$) were randomized to control and experimental groups. SBP was measured by a noninvasive tail-cuff method once per week for the duration of the experiment. Intact or castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice were infused with Ang II or its vehicle and injected with 6β-OHT (15 μg/g body weight, intraperitoneally, every third day) or its vehicle (dimethyl sulfoxide) for the duration of the experiment. Intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice were infused with Ang II or its vehicle and injected with 6β-OHT or testosterone (15 μg/g body weight, IP, every third day) or their vehicle (dimethyl sulfoxide) for the duration of the experiment. After 28 days of Ang II or vehicle infusion, abdominal aortas of the mice were analyzed for the development of AAAs by measuring the external aortic diameter with Vernier calipers. A maximal aortic diameter (MAD) of ≥1.5 mm (a 50% increase in diameter) was considered as an incidence of AAA. MADs of 1.5 to 1.9 mm were classified as Type 1 or less severe AAAs, and MADs >2 mm were classified as Type 2 or more severe AAAs.

Experimental Groups

A. *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice:

1. Vehicle (saline infusion)
2. Ang II (Ang II infusion)
3. 6β-OHT (saline infusion+6β-OHT treatment)
4. Ang II+6β-OHT (Ang II infusion+6β-OHT treatment)
5. Cas+Ang II (castrated+Ang II infusion)
6. Cas+Ang II+6β-OHT (castrated+Ang II infusion+6β-OHT treatment)

B. *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice:

1. Vehicle (saline infusion)
2. Ang II (Ang II infusion)
3. 6β-OHT (saline infusion+6β-OHT treatment)
4. Ang II+6β-OHT (Ang II infusion+6β-OHT treatment)
5. Testosterone (saline infusion+testosterone treatment)
6. Ang II+Testosterone (Ang II infusion+testosterone treatment)

Blood Pressure Measurement

SBP was measured every week by using a noninvasive tail-cuff method (model XBP 1000; Kent Scientific, Torrington, CT) for the duration of the experiment. Before implanting the mini-osmotic pumps, mice were acclimated to the blood pressure–measuring device for 1 week.

Histological and Immunohistochemical Analysis

At the completion of the experiments, mice were anesthetized as described above, and the animals were perfused with saline (3 minutes). The abdominal aortas were dissected and placed in Optimal Cutting Temperature compound (Sakura Finetek USA Inc., Torrance, CA) and frozen at −80°C. Aorta sections (5 μm) were sliced using a cryostat (model CM1850; Leica Microsystems, Bannockburn, IL) and stained for histological and immunohistochemical analysis. Briefly, sections were air-dried, fixed in 10% formalin or cold acetone for 10 minutes, washed with phosphate-buffered saline 3 times (3 minutes each), incubated with 0.3% hydrogen peroxide for 10 minutes to quench endogenous peroxides, washed with phosphate-buffered saline for 3 minutes, and blocked with serum appropriate for the species of the secondary antibody for 1 hour. Sections were then incubated with primary antibodies against α-actin (for smooth muscle cells) and CD68 (for macrophages), overnight at 4°C. The sections were then washed with phosphate-buffered saline 3 times (3 minutes each) and incubated with corresponding horseradish peroxidase–tagged or fluorophore-tagged secondary antibodies for 1 hour, for chromogenic detection of horseradish peroxidase activity with 3,3′-diaminobenzidine substrate or immunofluorescent detection, respectively. Also, sections were stained with hematoxylin and eosin, Masson's trichrome for collagen deposition, and Verhoeff–Van Gieson for elastin fibers, according to the manufacturer's instructions (Sigma, St Louis, MO). Sections were viewed in double-blinded fashion on an Olympus inverted system microscope (model BX41; Olympus America, Inc., Melville, NY) and photographed using a SPOT Insight digital camera (model Insight 2MP Firewire; Diagnostic Instruments, Inc., Sterling Heights, MI). While we were performing these studies, a Pannoramic scanner (3DHISTECH, Budapest, Hungary) became available to us, which we used for collagen detection in the aortic sections. Each slide containing 3 aorta sections/mouse was scanned using this scanner. Low- and high-magnification images were taken on the digitized slides using CaseViewer software (3DHISTECH). Three to 5 high-power images (×40) per aorta section per mouse were used to determine elastin degradation scoring according to a previously published method[18] as: grade 1, no degradation; grade 2, mild degradation; grade 3, severe degradation; and grade 4, aortic rupture. For smooth muscle α-actin, staining intensity (brown color) of 3 to

5 high-magnification (×40) images of each aorta section was quantified by colorimetric analysis using NIH ImageJ software and expressed as smooth muscle α-actin intensity using arbitrary unit per high-power field. Percentage of collagen accumulation in each aortic section was quantified using ImageJ.

Statistical Analysis

Data were analyzed by Fisher exact test, 1-way ANOVA, with Tukey's multiple comparisons test, and repeated-measures 2-way ANOVA, with Tukey's multiple comparisons test. The values of a minimum of 3 different experiments are expressed as the mean±SEM. $P<0.05$ were considered statistically significant. Power analysis was done using tools from Open Source Epidemiologic Statistics for Public Health (www.openepi.com). In most of the experiments, the primary outcomes and main comparisons exceeded a power of 80% with the number of animals used.

RESULTS

6β-OHT Treatment Restores Ang II-Induced AAA Incidence and Severity in Castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ Mice

To determine whether 6β-OHT, a metabolite of testosterone generated by CYP1B1,[19,20] contributes to AAA formation in male mice, we examined the effect of 6β-OHT administration on Ang II-induced AAA development in intact and castrated male *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice. Representative images of the excised aortas of mice from the different treatment groups are shown in Figure 1A and Figure S1. Subcutaneous infusion of Ang II for 28 days by osmotic minipumps was found to result in a significant increase in MAD (Figure 1B), compared saline-infused mice. A MAD of ≥1.5 mm was considered an incidence of AAA, with MADs of 1.5 to 1.9 mm classified as Type 1 or less severe AAAs, and MADs >2 mm as Type 2 or more severe AAAs. Saline infusion did not result in any AAA formation, and Ang II administration caused a 66.7% incidence of AAAs in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice (Figure 1C), of which 8.3% were Type 1 AAAs, and 58.3% were Type 2 AAAs (Figure 1D). As reported before,[10] castration of the male *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice significantly reduced MAD after 28 days of Ang II infusion compared with saline-infused mice (Figure 1B), resulting in a complete lack of AAA development (Figure 1C and 1D). However, treatment of castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice with 6β-OHT restored the Ang II-induced increase in MAD (Figure 1B), resulting in a higher incidence (50%; Figure 1C) as well as severity (21.4% Type 1 and 28.6% Type 2; Figure 1D) of AAAs than those observed in Ang II-treated castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice. Treatment with 6β-OHT alone (without Ang II) did not result in AAA formation in these mice.

6β-OHT Treatment Restores Ang II-Induced AAA Incidence and Severity in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ Mice

Previously we reported that *Cyp1b1* gene disruption attenuated AAA formation in male *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice.[13] Since Ang II stimulates the production of 6β-OHT in *Cyp1b1*$^{+/+}$ but not in *Cyp1b1*$^{-/-}$ male mice,[14] we investigated the effect of 6β-OHT on Ang II-induced AAA development in male *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. Representative images of the excised aortas of mice from the different treatment groups are shown in Figure 2A and Figure S1. Ang II infusion did not increase MAD compared with saline infusion (Figure 2B), resulting in a 0% incidence of AAAs in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice (Figure 2C), confirming our previous report.[13] However, administration of 6β-OHT restored the Ang II-induced increase in MAD (Figure 2B), leading to a 62.5% incidence of AAA formation (Figure 2C), similar to the AAA percentage incidence observed in Ang II-infused castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice (Figure 1B). These AAAs comprised an equal percentage (31.3%) of the less severe Type 1 and the more severe Type 2 AAAs (Figure 2D). In contrast, administration of testosterone, along with Ang II, failed to increase the MAD (Figure 2B), incidence (10%; Figure 2C), as well as the severity of Ang II-induced AAAs in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. Treatments with 6β-OHT or testosterone, in the absence of Ang II infusion, did not increase MAD or result in AAA formation in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice (Figure 2B and 2C).

Castration or *Cyp1b1* Gene Disruption Attenuates Ang II-Induced Histopathological Features of AAAs in *Apoe*$^{-/-}$ Mice, Which Is Restored by 6β-OHT Administration

Histopathological analysis of abdominal aortas after hematoxylin and eosin staining revealed vascular AAA development in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice that were infused with Ang II for 28 days (Figure 3A). While castration alone did not result in Ang II-induced AAA development in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, administration of 6β-OHT to castrated mice resulted in extensive AAA expansion after 28 days of Ang II infusion. These AAAs were found to demonstrate the usual aneurysmal pathology including extensive elastin straightening/disorganization, disruption, and degradation, in contrast to the typical wavy appearance of elastin fibers in the aortas of saline-infused intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and Ang II-infused castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice (Figure 3B). In addition, in 6β-OHT-treated Ang II-infused castrated

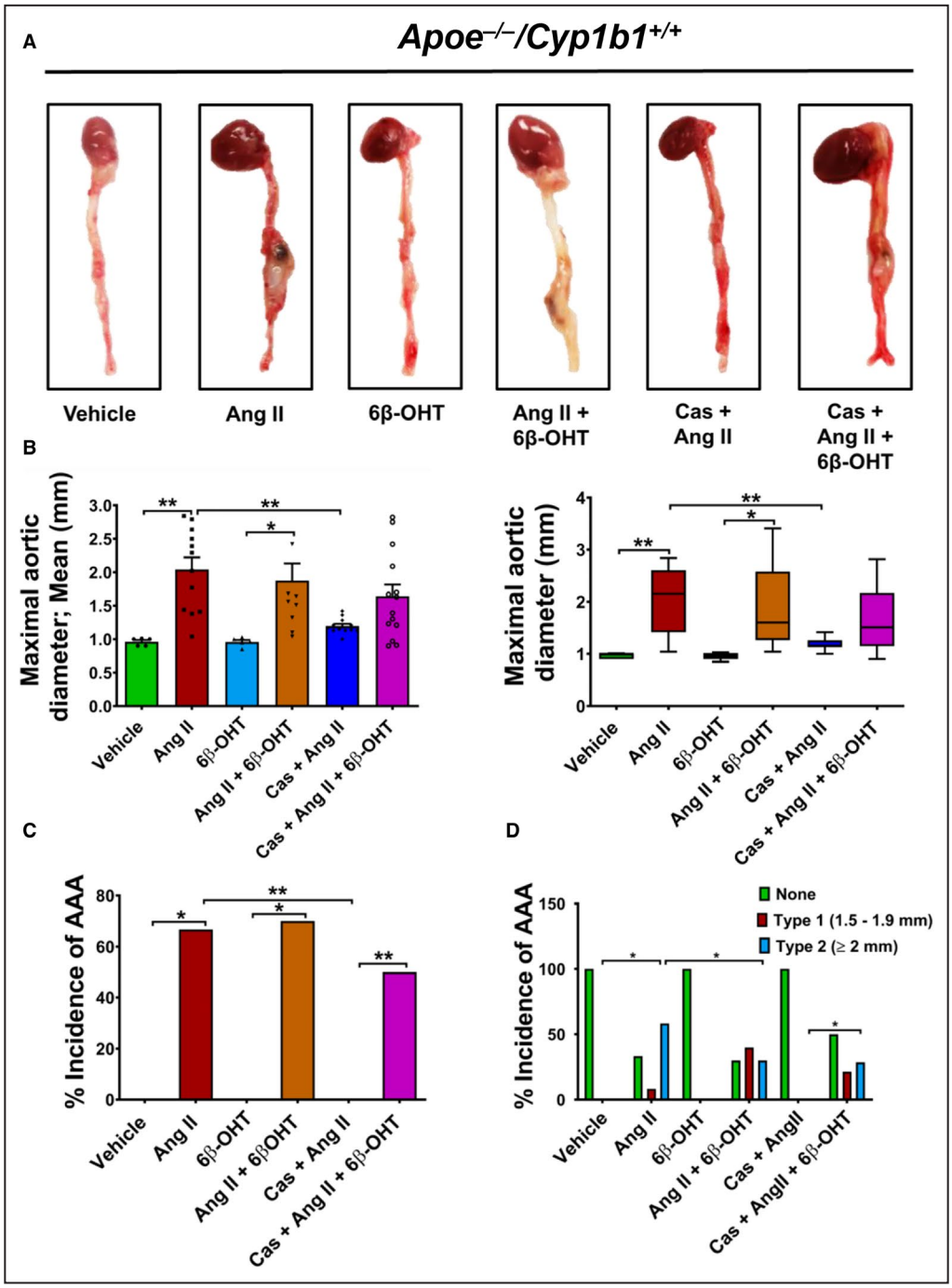

Figure 1. 6β-OHT treatment restores Ang II-induced AAA incidence and severity in castrated *Apoe*$^{-/-}$ mice.

Representative images of aorta (**A**); maximal aortic diameter (**B**: mean [left]; maximum–minimum [right]); incidence of AAAs (**C**) and severity of AAAs (**D**) in intact or castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice infused with Ang II or its vehicle (saline) for 28 days and administered 6β-OHT or its vehicle DMSO; n=5 for Vehicle group, n=12 for Ang II group, n=5 for 6β-OHT group, n=10 for Ang II+6β-OHT group, n=12 for Cas+Ang II group, n=14 for Cas+Ang II+6β-OHT group. Data are expressed as mean±SEM and analyzed using 1-way ANOVA, with Tukey's multiple comparisons test (for aortic diameter) and Fisher exact test (for AAA incidence); *P<0.05; **P<0.01. AAA indicates abdominal aortic aneurysm; Ang II, angiotensin II; Cas, castrated; *Cyp1b1*, cytochrome P450 1b1; DMSO, dimethyl sulfoxide; and 6β-OHT, 6β-hydroxytestosterone.

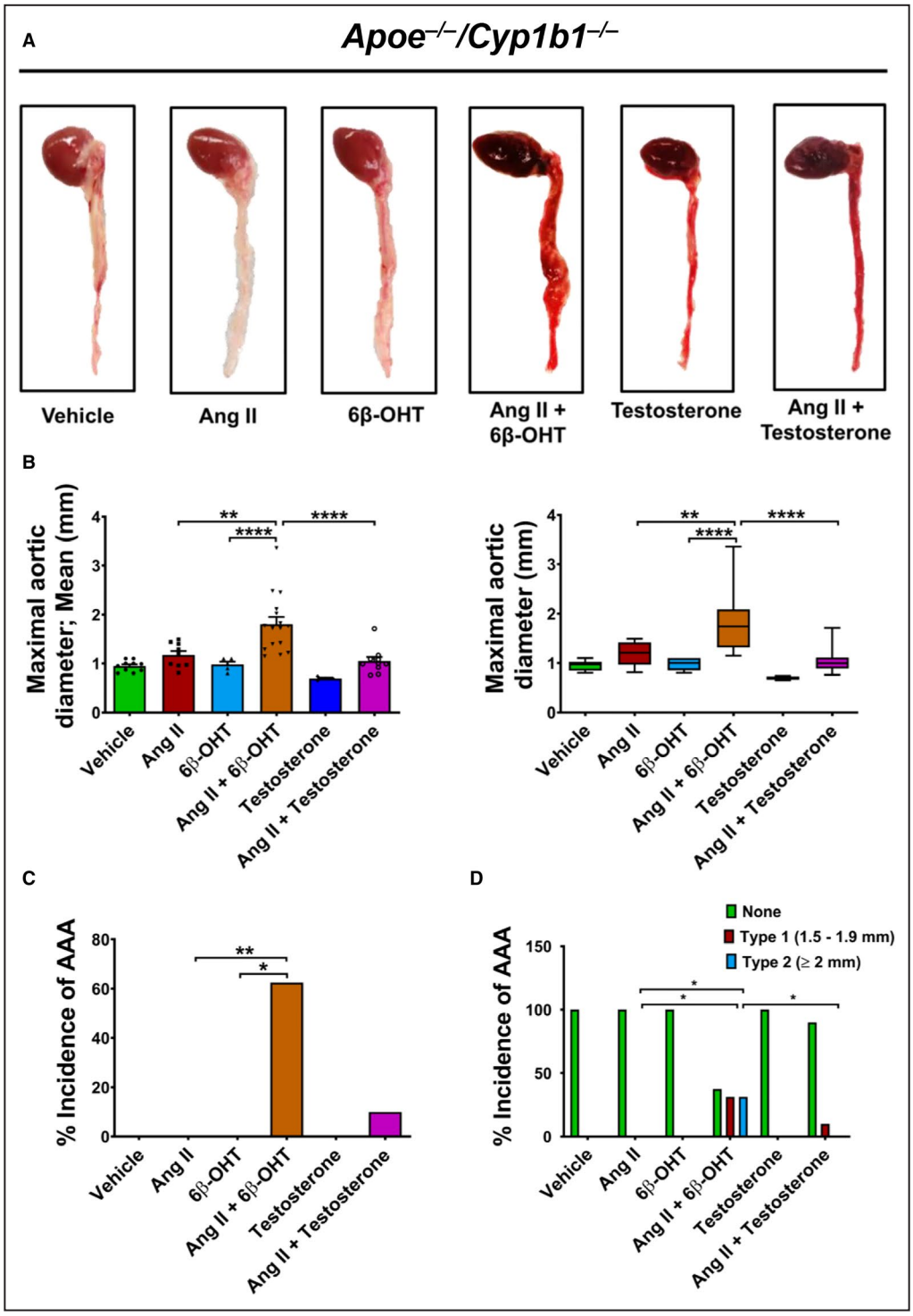

Figure 2. 6β-OHT treatment restores Ang II-induced AAA incidence and severity in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice.

Representative images of aorta (**A**); maximal aortic diameter (**B**: mean [left]; maximum-minimum [right]); incidence of AAAs (**C**) and severity of AAAs (**D**) in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice infused with Ang II or its vehicle (saline) for 28 days and administered 6β-OHT or testosterone or their vehicle DMSO; n=10 for Vehicle group, n=9 for Ang II group, n=5 for 6β-OHT group, n=16 for Ang II+6β-OHT group, n=4 for Testosterone group, n=10 for Ang II+Testosterone group. Data are expressed as mean±SEM and analyzed using 1-way ANOVA, with Tukey's multiple comparisons test (for aortic diameter) and Fisher exact test (for AAA incidence); **P*<0.05; ***P*<0.01; *****P*<0.0001. AAA indicates abdominal aortic aneurysm; Ang II, angiotensin II; *Cyp1b1*, cytochrome P450 1b1; DMSO, dimethyl sulfoxide; and 6β-OHT, 6β-hydroxytestosterone.

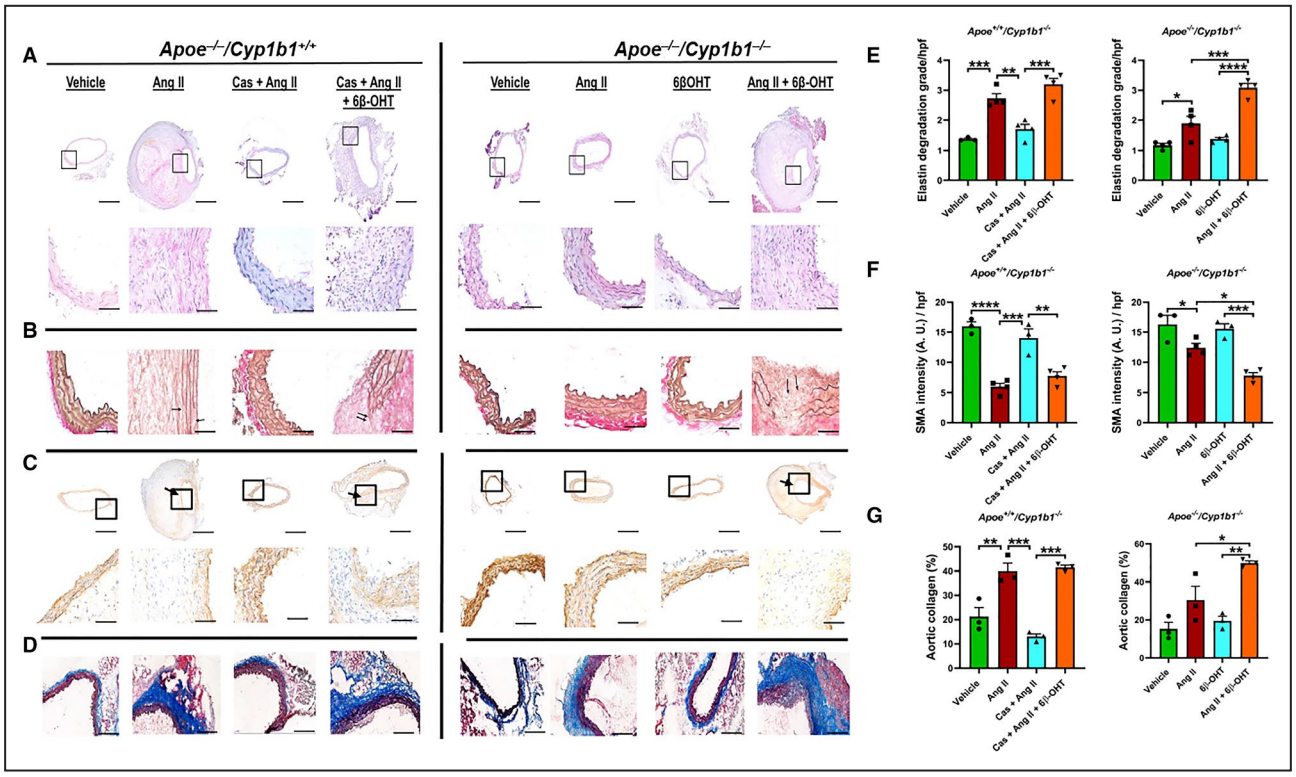

Figure 3. Castration or *Cyp1b1* gene disruption attenuates Ang II-induced histopathological features of AAAs in *Apoe*$^{-/-}$ mice, which is restored by 6β-OHT administration.

Representative images of H&E staining (**A**), VVG staining of elastin fibers (**B**), α-actin expression (brown) (**C**; arrows indicate attenuated α-actin expression in medial layers adjacent to the aneurysmal lesion), and Masson's trichrome staining of collagen (collagen: blue; smooth muscle: red) (**D**) in abdominal aortas of *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. Scale bars represent 200 μm (upper panel), 20 μm (lower panel) for H&E, elastin and α-actin, and 50 μm for collagen. Quantitation of elastin degradation grade (**E**), smooth muscle α-actin (SMA) intensity (**F**) and aortic collagen (**G**) in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. n=3 to 4 in each group. Data are expressed as mean±SEM and analyzed using 1-way ANOVA with Tukey's multiple comparison test; **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. AAA indicates abdominal aortic aneurysm; Ang II, angiotensin II; Cas, castrated; *Cyp1b1*, cytochrome P450 1b1; H&E, hematoxylin and eosin; 6β-OHT, 6β-hydroxytestosterone; and VVG, Verhoeff–Van Gieson.

Apoe$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, abundant collagen deposition, suggestive of an altered smooth muscle cell phenotype, was found in pockets of the aneurysmal tissue (Figure 3D). However, attenuated collagen (Figure 3D), as well as α-actin expression (Figure 3C), indicative of loss of extracellular matrix (ECM) integrity, was observed in the medial layers adjacent to the lesion, as well as within the necrotic regions. In contrast, Ang II in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice failed to produce any AAA expansion (Figure 3A) or associated pathologies such as elastin damage or ECM modification. However, the administration of 6β-OHT after Ang II infusion in the *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice restored the aneurysmal pathological phenotype (Figure 3A) with extensive elastin fiber degradation (Figure 3B), collagen modifications, and attenuated α-actin expression (Figure 3C and 3D). The representative images of hematoxylin and eosin staining, elastin, α-actin and collagen, and quantitation of the changes in various treatment groups are shown in Figure 3A through 3G. The additional images of these histological markers are shown in Figures S2 through S5.

Castration or *Cyp1b1* Gene Disruption Prevents Inflammatory Cell Infiltration in Ang II-Induced AAAs in *Apoe*$^{-/-}$ Mice, Which Is Restored by 6β-OHT Administration

To determine the contribution of the CYP1B1-generated testosterone metabolite 6β-OHT in inflammatory cell infiltration associated with Ang II-induced AAAs, we examined the localization of CD68^{+} macrophages in the aortas of Ang II-infused intact and castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice, administered with 6β-OHT or its vehicle. Ang II infusion significantly increased expression of CD68^{+} macrophages in the aneurysmal tissue of intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, which was abrogated in Ang II-infused aortas of castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/

Cyp1b1$^{-/-}$ mice. However, administration of 6β-OHT to Ang II-infused castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice restored infiltration of inflammatory cells into aortas during AAA development, as evidenced by positive staining of CD68 within aneurysm tissue (Figure 4A and 4B). The large size images of the CD68 immunofluorescent staining for each group for better visualization are shown in Figure S6.

Castration or *Cyp1b1* Gene Disruption Attenuates Ang II-Induced Increase in SBP in *Apoe*$^{-/-}$ Mice, Which Is Restored by 6β-OHT Administration

Ang II infusion is associated with several cardiovascular pathologies, including hypertension. Ang II infusion for 28 days increased SBP in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, starting 7 days after Ang II administration, and remained elevated at all other measured time-points (days 14, 21, and 28 after Ang II infusion) (Figure 5A). However, it reached statistical significance only on Day 14. Castration of *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice or *Cyp1b1* gene disruption minimized this increase in SBP (Figure 5A and 5B). However, the administration of 6β-OHT in castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ as well as intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice restored the ability of Ang II to increase SBP to levels similar to those observed in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice (Figure 5A and 5B).

DISCUSSION

Male sex is an established nonmodifiable risk factor for AAA development. A 2- to 10-fold higher prevalence of AAAs is observed in males than females, with overall rates of AAA ranging from 1.9% to 18.5% in males and 0% to 4.2% in females.[2,10,21] While several factors, such as aortic stiffness,[22] hemodynamics, and aortic size,[23–25] and more recently, sex chromosomes[8,26] were shown to play a role, sex hormones have been widely studied to better understand their role in the development of AAAs.[5] This study demonstrates, for the first time, that 6β-OHT, a metabolite of testosterone generated by CYP1B1, is essential for Ang II-induced AAAs and hypertension development in male mice. The key findings of this study are the following: (1) Ang II increased the incidence, severity, and pathological hallmarks of AAAs in the intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, which were minimized in castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice, as previously reported.[10,13] (2) Administration of 6β-OHT restored the incidence, severity, and associated pathological changes in Ang II-induced AAAs in castrated

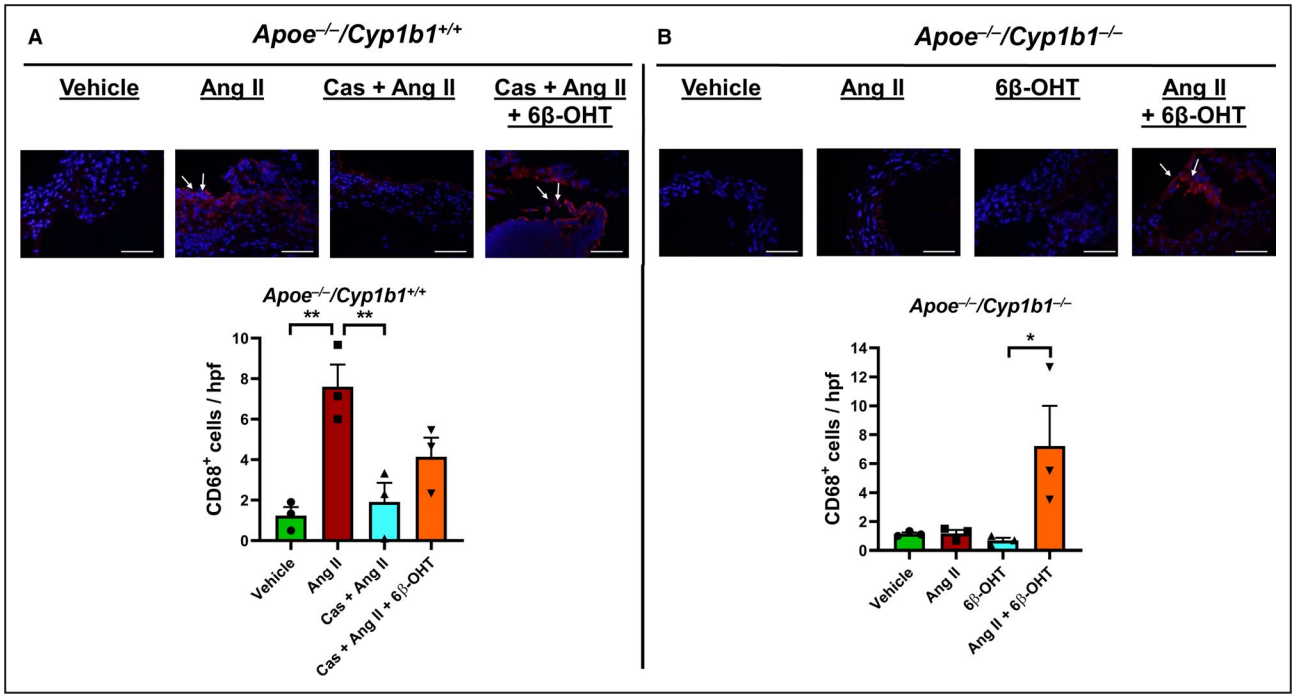

Figure 4. Castration or *Cyp1b1* gene disruption prevents inflammatory cell infiltration in Ang II-induced AAAs in *Apoe*$^{-/-}$ mice, which is restored by 6β-OHT administration.
Representative images of infiltration of CD68^{+} macrophages (red) and nuclear staining with 4′,6-diamidino-2-phenylindole (blue) and quantitative analysis of total number of CD68^{+} macrophages per high-power field (hpf) in abdominal aortas of (**A**) *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and (**B**) *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. Arrows indicate CD68^{+} macrophages. Scale bars represent 20 μm. n=3 in each group. Data are expressed as mean±SEM and analyzed using 1-way ANOVA with Tukey's multiple comparison test; **P*<0.05; ***P*<0.01. Ang II indicates angiotensin II; Cas, castrated; *Cyp1b1*, cytochrome P450 1b1; and 6β-OHT, 6β-hydroxytestosterone.

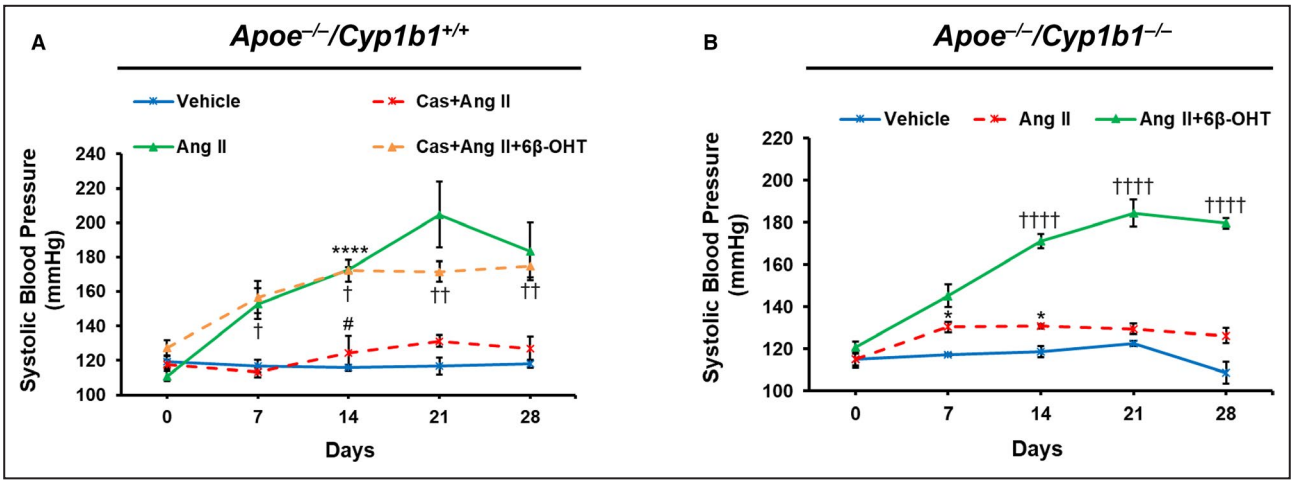

Figure 5. Castration or *Cyp1b1* gene disruption attenuates Ang II-induced increase in systolic blood pressure in *Apoe*$^{-/-}$ mice, which is restored by 6β-OHT administration.
Systolic blood pressure during Ang II infusion in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ (**A**) and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ (**B**) mice; (**A**) ****P<0.0001, Vehicle vs Ang II; $^{\#}P$<0.05 Ang II vs Cas+Ang II; $^{\dagger}P$<0.05; $^{\dagger\dagger}P$<0.01, Cas+Ang II vs Cas+Ang II+6β-OHT; (**B**) *P<0.05, Vehicle vs Ang II; $^{\dagger\dagger\dagger\dagger}P$<0.0001 Ang II vs Ang II+6β-OHT. For *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice: n=4 for Vehicle group, n=3 for Ang II group, n=5 for Cas+Ang II group, n=5 for Cas+Ang II+6β-OHT group; for *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice: n=4 for Vehicle group, n=5 for Ang II group, n=8 for Ang II+6β-OHT group. Data are expressed as mean±SEM and analyzed using repeated-measures 2-way ANOVA, with Tukey's multiple comparisons test. Ang II indicates angiotensin II; Cas, castrated; *Cyp1b1*, cytochrome P450 1b1; and 6β-OHT, 6β-hydroxytestosterone.

Apoe$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. (3) In contrast, treatment with testosterone failed to restore incidence and severity of Ang II-induced AAAs in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. (4) Administration of 6β-OHT restored the Ang II-induced increase in SBP in both castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice.

Ang II infusion caused the development of AAAs in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ male mice, which were minimized by castration and reversed by dihydrotestosterone.[7,10,27] However, in the present study, CYP1B1 gene disruption in *Apoe*$^{-/-}$ male mice also abrogated Ang II-induced AAAs, confirming our previous observation.[13] Testosterone can also be metabolized by CYP1B1 into 6β-OHT.[19,20] Although our current study in *Apoe*$^{-/-}$ mice is limited in not measuring testosterone and its metabolites, we have previously reported that Ang II stimulates the production of 6β-OHT but not dihydrotestosterone in *Cyp1b1*$^{+/+}$ mice, which is abolished in *Cyp1b1*$^{-/-}$ mice.[14] These observations and our demonstration that in the castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, treatment with 6β-OHT abrogated the protective effect of castration to minimize the incidence and severity of AAAs, suggest that this CYP1B-testosterone-generated metabolite mediates Ang II-induced AAAs in male mice. Supporting this conclusion was our finding that the administration of 6β-OHT to *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice that lacked endogenous 6β-OHT[14] also restored the effect of Ang II to produce AAAs. Moreover, administration of testosterone alone or with Ang II failed to produce AAAs in *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice, suggesting that metabolism of testosterone to 6β-OHT by CYP1B1 is a critical step for Ang II-induced AAA formation. A cross-sectional study of AAAs in affected patients reported lower free and total testosterone levels than in men without AAAs, which also correlated inversely with AAA.[28] Whether or not the age-related decline in testosterone levels[29] and the associated increased prevalence of AAA with advanced age[1,2] is because of increased metabolism of testosterone to 6β-OHT by CYP1B1 remains to be determined. The contribution of the testosterone-CYP1B1-generated metabolite 6β-OHT in the development of AAA aneurysms could also be dependent on background levels of Ang II and agents that increase CYP1B1 activity. We previously found that Ang II increases cardiac CYP1B1 activity without altering CYP1B1 expression in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice.[13] Whether CYP1B1 expression and/or activity is increased in the abdominal aorta during AAA development across different regions of the aorta remains to be determined.

Elastin, collagen, and proteoglycans are the key macromolecules of the ECM and impart biomechanical properties to the vessel wall.[6] AAA is characterized by progressive dilation of the abdominal aorta over years or decades because of remodeling of the ECM and structural degradation of the arterial wall.[6,30] However, disease progression from initiation to final pathology is thought to be a heterogeneous process involving ECM changes at different stages in AAA development. The initial stage of aortic dilation is primarily characterized by elastin degradation and collagen production, followed by gradual expansion involving a delicate balance between collagen production and

degradation, and finally, a stage of rapid expansion and rupture because of accelerated collagen degradation exceeding the repair process.[30] This remodeling pattern was reflected in the results of our AAA histological analysis, which showed that treatment with Ang II in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice led to extensive elastin fiber straightening/degradation and an increased volume, but less intense collagen deposition, in the area of AAAs. Since these histological changes associated with Ang II-induced AAAs were minimized in castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice, but restored by treatment with 6β-OHT, these results support our contention that this testosterone metabolite mediates the vascular remodeling observed in AAAs. These results, coupled with our previous findings in mouse models of atherosclerosis,[17] AAAs,[13] and neointimal growth,[31] imply that CYP1B1 plays an important role in elastin and collagen remodeling within blood vessels. However, although aortic dilation, including disruption of elastic fibers, has been attributed to sex hormones,[6,32] we did not find any direct effect of 6β-OHT and/or testosterone on ECM remodeling in the absence of Ang II. Therefore, from these observations, it follows that vascular remodeling associated with Ang II-induced AAAs requires the testosterone-CYP1B1-generated metabolite 6β-OHT in *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice.

Changes in smooth muscle cell (SMC) phenotype from a contractile/differentiated to a synthetic/dedifferentiated phenotype is a pathological hallmark of several cardiovascular diseases,[33–35] including AAAs.[1] α-Actin is a prominent marker of differentiated SMCs, and is known to be decreased in aneurysmal lesions.[1,35] Our current results showed reduced expression of α-actin in the medial layers adjacent to the aneurysmal lesion, suggestive of phenotypic switching and structural loss of medial SMCs during Ang II-induced AAA development in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice. These changes in the structure of AAAs were attenuated in the castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice, and restored by administration of 6β-OHT. This change in the SMC phenotype is known to be regulated by cyclooxygenase-2, an enzyme in the arachidonic acid metabolism pathway, during Ang II-induced AAA progression.[1] Also, we previously reported that CYP1B1, via the metabolism of arachidonic acid and generation of reactive oxygen species, is essential for Ang II-induced vascular SMC proliferation and migration, properties exhibited by dedifferentiated SMCs in vitro.[31,36] However, the mechanism by which the CYP1B1-generated metabolite of testosterone, 6β-OHT, regulates phenotypic switching of SMCs associated with Ang II-induced AAAs in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice, especially considering its important role in cardiovascular diseases, is a critical question and remains to be addressed.

Inflammatory cell infiltration into the wall of the abdominal aorta is 1 of the key initiating factors of Ang II-induced AAA development.[37] Macrophages are the most common type of inflammatory cells known to accumulate in the medial layers as well as adventitial walls from the initial to the most advanced stages of Ang II-induced AAAs.[38–40] As reported previously,[13] the infiltration of CD68^{+} macrophages, which are markers of inflammation in Ang II-induced AAAs in male mice, was dependent on CYP1B1. In the present study, the infiltration of CD68^{+} macrophages in the area of AAAs caused by Ang II in intact *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ mice that were minimized in the castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice was restored by treatment with 6β-OHT. These observations support our hypothesis that 6β-OHT contributes to the pathogenesis of Ang II-induced AAAs in male mice. Since CYP1B1 is also expressed in macrophages,[41] further studies utilizing a macrophage-specific conditional knockout mouse model are required to determine whether CYP1B1 via the testosterone-generated metabolite 6β-OHT in this cell type contributes to Ang II-induced AAA formation. One could also perceive some cross-talk between CYP1B1 and other arachidonic acid–metabolizing enzymes involved in the inflammatory cascade during AAA development. As discussed above, genetic disruption and/or pharmacological inhibition of cyclooxygenase-2, a major arachidonic acid–metabolizing enzyme, attenuated Ang II-induced AAA development.[1,38,42] Antagonism of the leukotriene pathway was also shown to decrease AAA formation.[43–45] However, whether all these 3 arachidonic acid metabolic pathways act sequentially, in parallel, or a redundant fashion, remains to be investigated.

Finally, we found that the administration of 6β-OHT restored the Ang II-induced increase in SBP in castrated *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and intact *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice. These results confirm our previous reports on the contribution of 6β-OHT to Ang II-induced hypertension in *Cyp1b1*$^{-/-}$ mice.[14] Although we have not measured lipid levels in these mice, extension of the findings from *Apoe*$^{+/+}$ to *Apoe*$^{-/-}$ background as well as with prolonged Ang II infusion period (14–28 days) is crucial, especially considering the potential use of CYP1B1 inhibitors as antihypertensive agents to treat men with dyslipidemia. It is well established that Ang II-induced AAA formation in *Apoe*$^{-/-}$ mice is independent of increased blood pressure.[46] Therefore, we believe that the development of Ang II-induced AAAs mediated by testosterone-CYP1B1-generated 6β-OHT is also independent of hypertension.

The mechanism by which 6β-OHT contributes to the effects of Ang II to facilitate AAA development remains to be determined. Inflammatory cells are intricately involved in AAA development,[37] including in our

current study, where we observed robust infiltration of CD68+ macrophages in the aneurysmal lesions. Also, matrix metalloproteinases are critical modulators of Ang II-induced AAA development.[47] We have previously reported the role of matrix metalloproteinases in CYP1B1-dependent AAA formation in mice.[13] In this regard, caveolin-1, in vascular SMCs, which mediates epidermal growth factor receptor transactivation via the metalloproteinase ADAM17 (a disintegrin and metalloprotease 17), is an important signaling molecule in AAA formation.[48] Considering our previous findings that CYP1B1-generated oxidative stress is an important contributor in cardiovascular disease, including AAAs,[12–16] CYP1B1-generated 6β-OHT might promote Ang II-induced AAA via caveolin and matrix metalloproteinase–dependent oxidative stress in *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$ mice. We previously reported that Ang II increases ERK1/2 (extracellular-signal-regulated kinase) and p38 MAPK (mitogen-activated protein kinase) activity in the aorta as well as in cultured rat vascular SMCs, which was attenuated by treatment with the CYP1B1 inhibitor 2,3′,4,5′-tetramethoxystilbene.[12,36] These signaling pathways are also well-established as playing an important role in AAA formation.[49] Therefore, further studies are required to determine whether ERK1/2 and p38 MAPK contribute to the effect of 6β-OHT in promoting Ang II-induced AAA development.

In conclusion, the present study demonstrates that the CYP1B1-generated metabolite of testosterone, 6β-OHT, contributes to Ang II-induced AAAs and associated pathogenesis and hypertension *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$, a model of hyperlipidemic male mice. Therefore, inhibitors of CYP1B1 could be useful for treatment of AAAs and hypertension in males with hyperlipidemia and high levels of Ang II, and those having testosterone supplements with cardiovascular disease.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Figures S1–S6

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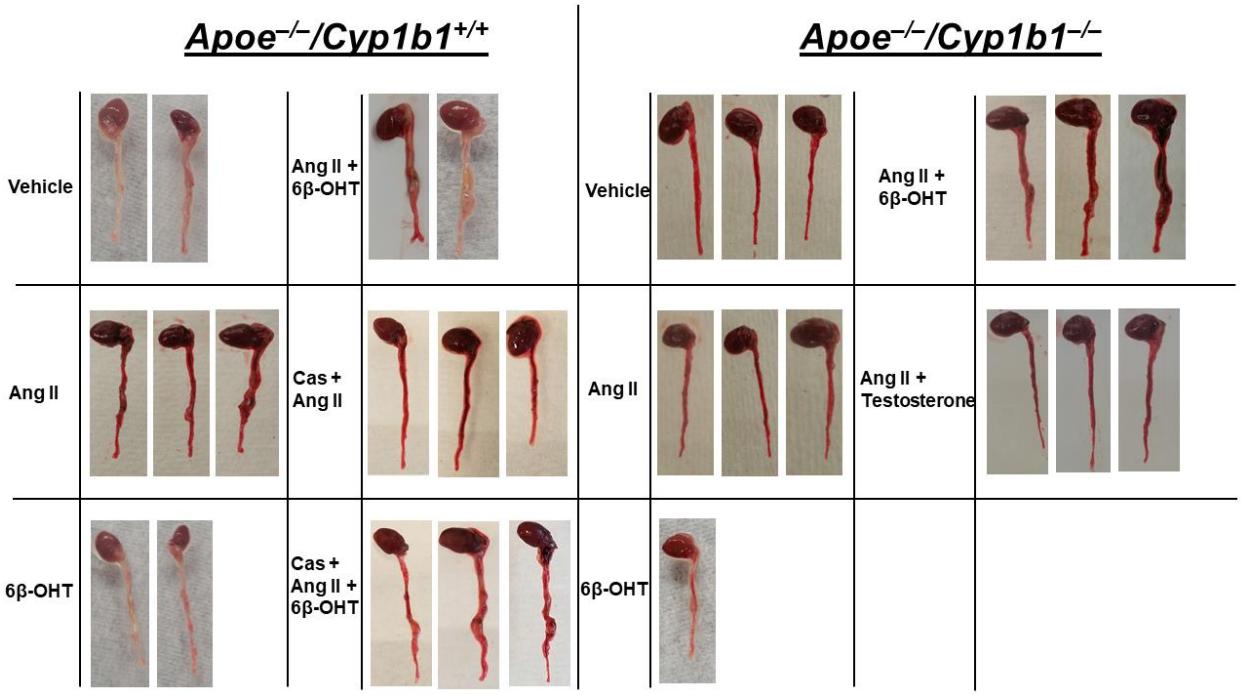
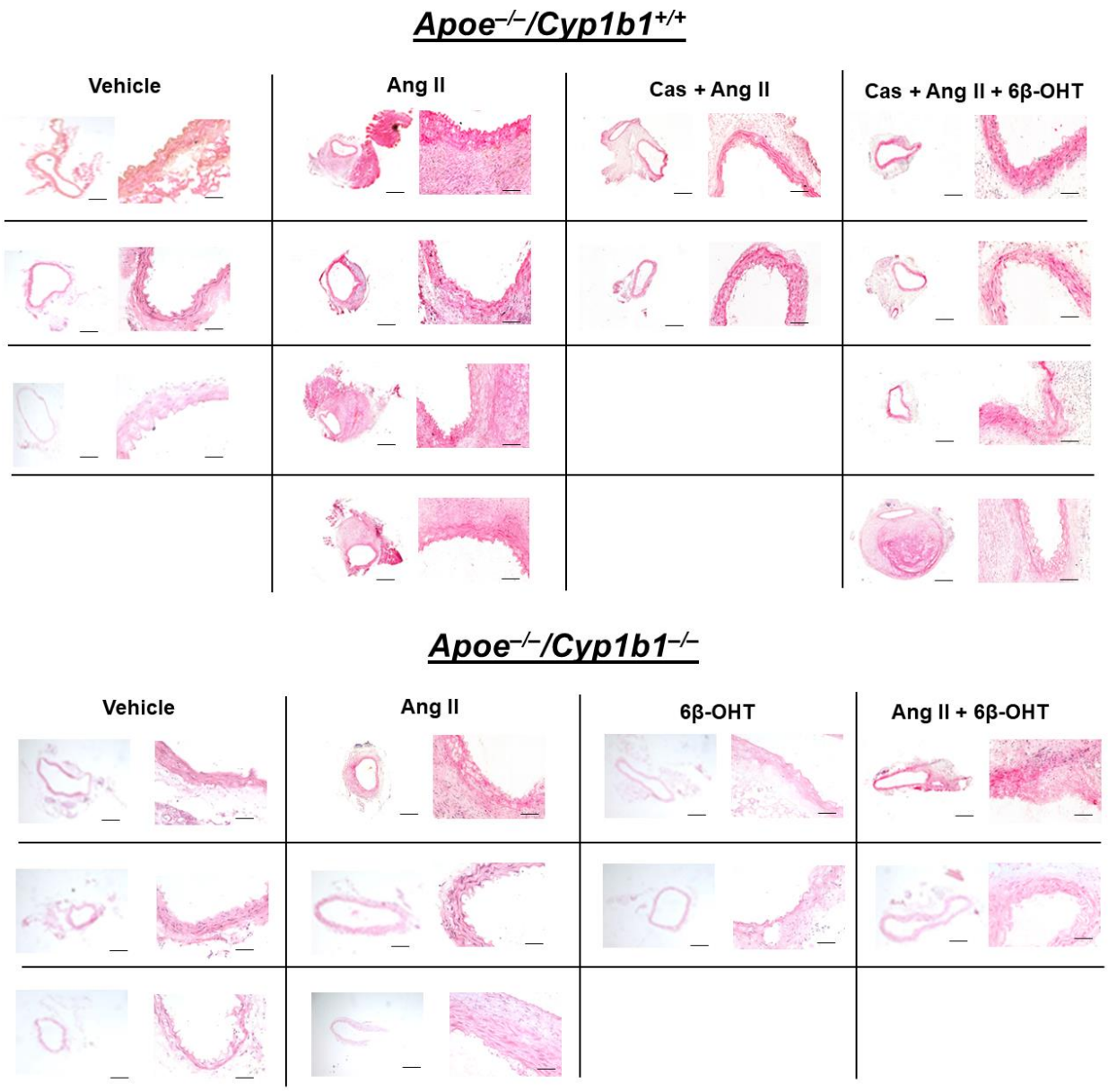
SUPPLEMENTAL MATERIAL

Figure S1. Representative *ex vivo* images of aorta of *Apoe*$^{-/-}$/*Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$/*Cyp1b1*$^{-/-}$ mice.



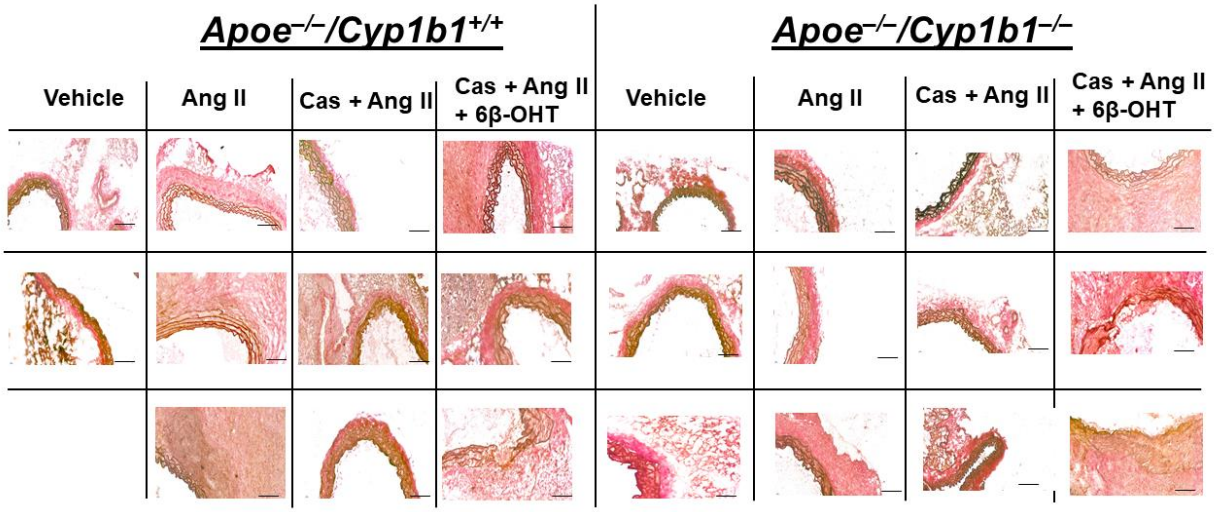
Cyp1b1, cytochrome P450 1b1; Ang II, angiotensin II; 6β-OHT, 6β-hydroxytestosterone; Cas, castrated

Figure S2. H&E staining depicting histopathological features of abdominal aortas



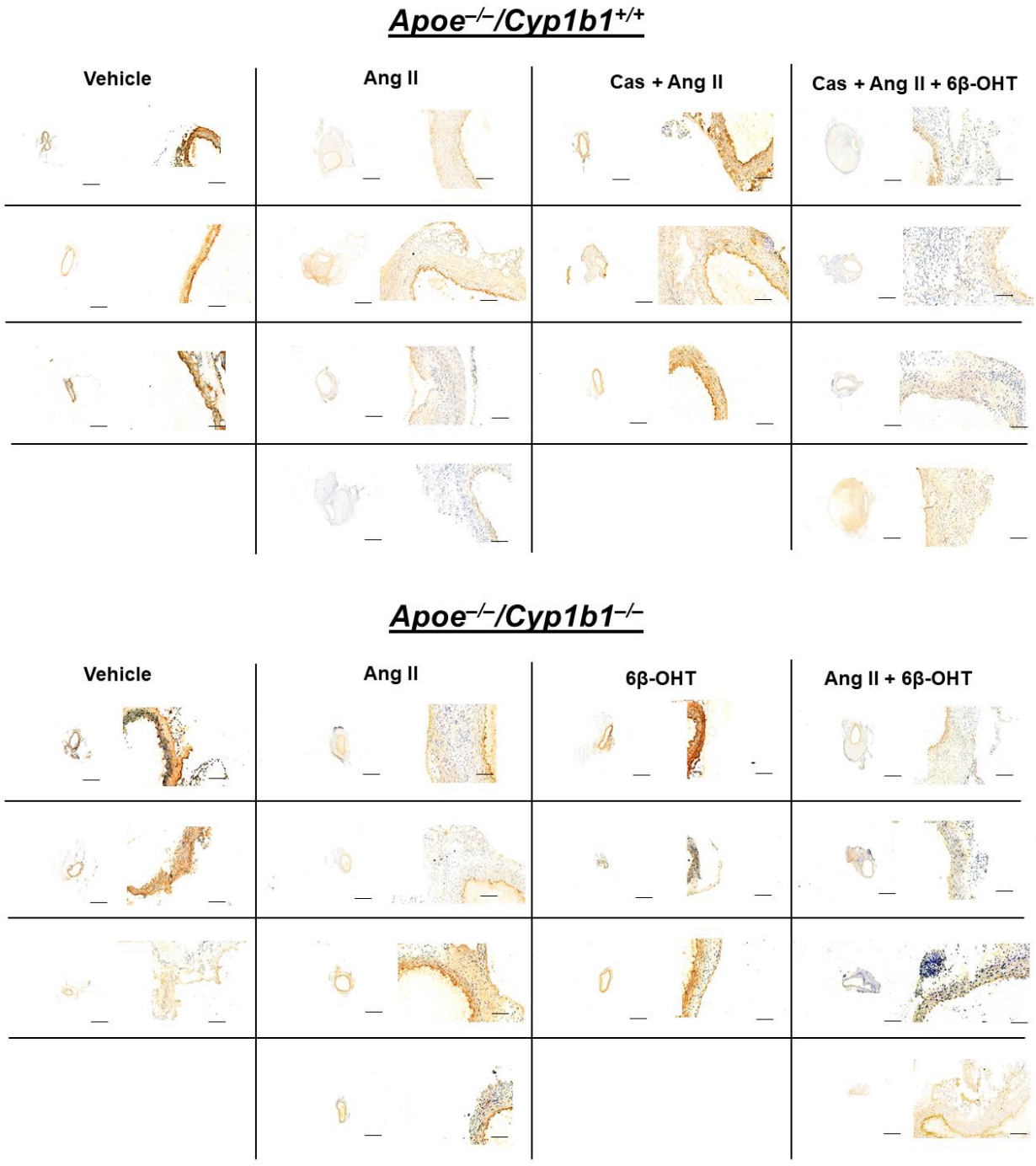
H&E stained images of abdominal aortas of *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$*/Cyp1b1*$^{-/-}$ mice. Scale bars represent 200 μm (left panel), and 20 μm (right panel). H&E, hematoxylin and eosin; *Cyp1b1*, cytochrome P450 1b1; Ang II, angiotensin II; 6β-OHT, 6β-hydroxytestosterone; Cas, castrated

Figure S3. VVG staining depicting elastin fibers of abdominal aortas



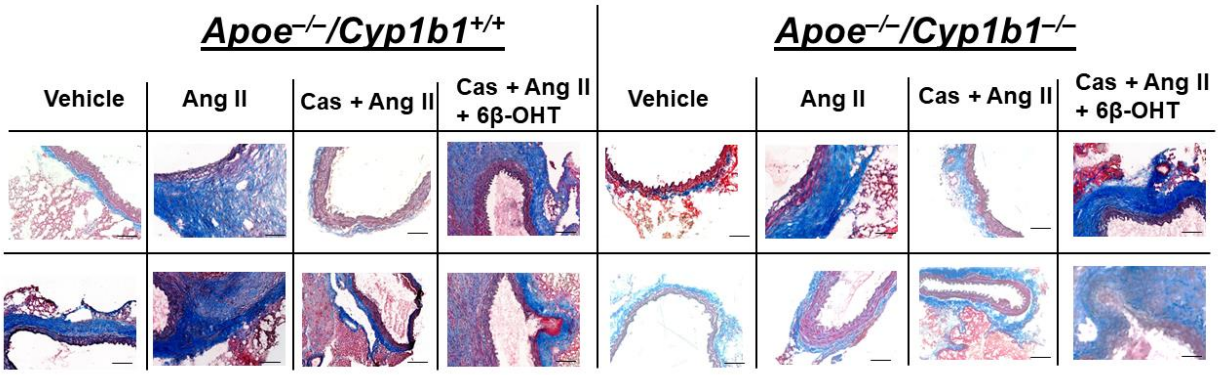
VVG stained images of abdominal aortas of *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$*/Cyp1b1*$^{-/-}$ mice. Scale bars represent 20 μm. VVG, Verhoeff–Van Gieson; *Cyp1b1*, cytochrome P450 1b1; Ang II, angiotensin II; 6β-OHT, 6β-hydroxytestosterone; Cas, castrated

Figure S4. α-actin expression in abdominal aortas.



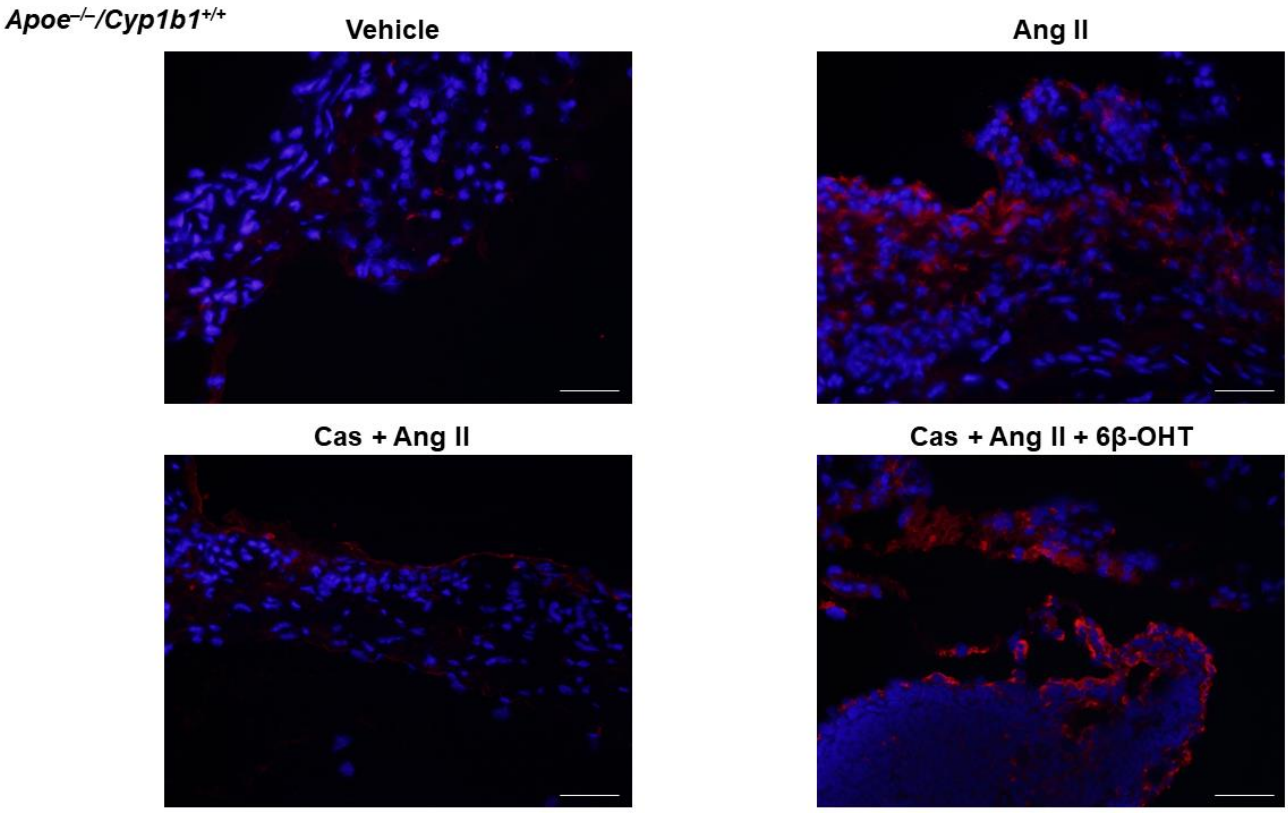
Immunohistochemistry depicting α-actin expression in abdominal aortas of *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$*/Cyp1b1*$^{-/-}$ mice. Scale bars represent 200 µm (left panel), and 20 µm (right panel). *Cyp1b1*, cytochrome P450 1b1; Ang II, angiotensin II; 6β-OHT, 6β-hydroxytestosterone; Cas, castrated

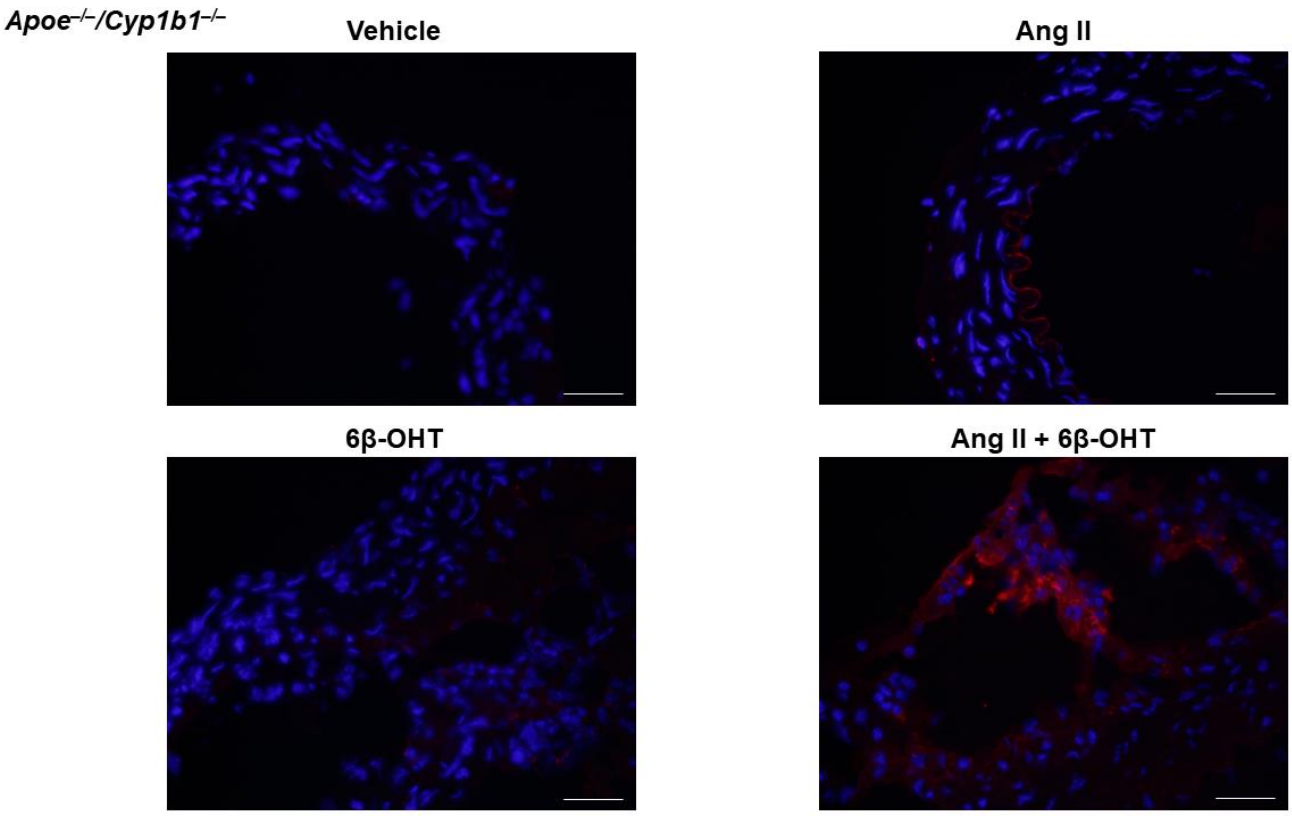
Figure S5. Collagen expression in abdominal aortas.



Masson's trichrome staining of collagen (collagen: blue; smooth muscle: red) of abdominal aortas of *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$*/Cyp1b1*$^{-/-}$ mice. Scale bars represent 50 μm. *Cyp1b1*, cytochrome P450 1b1; Ang II, angiotensin II; 6β-OHT, 6β-hydroxytestosterone; Cas, castrated

Figure S6. Castration or *Cyp1b1* gene disruption prevents inflammatory cell infiltration in Ang II-induced AAAs in *Apoe*$^{-/-}$ mice, which is restored by 6β-OHT administration





Representative magnified images (of Figure 4) of infiltration of $CD68^+$ macrophages (red) and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (blue) in abdominal aortas of *Apoe*$^{-/-}$*/Cyp1b1*$^{+/+}$ and *Apoe*$^{-/-}$*/Cyp1b1*$^{-/-}$ mice. Scale bars represent 20 µm. *Cyp1b1*, cytochrome P450 1b1; Ang II, angiotensin II; 6β-OHT, 6β-hydroxytestosterone; Cas, castrated