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Cyp2c44 gene disruption exacerbated pulmonary hypertension and heart failure in female but not male mice

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Abstract: Epoxyeicosatrienoicacids (EETs), synthesized from arachidonic acid by epoxygenases of the CYP2C and CYP2J gene subfamilies, contribute to hypoxic pulmonary vasoconstriction (HPV) in mice. Despite their roles in HPV, it is controversial whether EETs mediate or ameliorate pulmonary hypertension (PH). A recent study showed that deficiency of *Cyp2j* did not protect male and female mice from hypoxia-induced PH. Since CYP2C44 is a functionally important epoxygenase, we hypothesized that knockout of the *Cyp2c44* gene would protect both sexes of mice from hypoxia-induced PH. We tested this hypothesis in wild-type (WT) and *Cyp2c44* knockout ($Cyp2c44^{-/-}$) mice exposed to normoxia (room air) and hypoxia (10% O_2) for 5 weeks. Exposure of WT and $Cyp2c44^{-/-}$ mice to hypoxia resulted in pulmonary vascular remodeling, increased pulmonary artery resistance, and decreased cardiac function in both sexes. However, in female $Cyp2c44^{-/-}$ mice, compared with WT mice, (1) pulmonary artery resistance and right ventricular hypertrophy were greater, (2) cardiac index was lower, (3) left ventricular and arterial stiffness were higher, and (4) plasma aldosterone levels were higher, but (5) there was no difference in levels of EET in lungs and heart. Paradoxically and unexpectedly, we found that *Cyp2c44* disruption exacerbated hypoxia-induced PH in female but not male mice. We attribute exacerbated PH in female $Cyp2c44^{-/-}$ mice to elevated aldosterone and as-yet-unknown systemic factors. Therefore, we suggest a role for the human *CYP2C* genes in protecting women from severe PH and that this could be one of the underlying causes for a better 5-year survival rate in women than in men.

Keywords: arachidonic acid, cytochrome P450, aldosterone, sex, vascular remodeling.

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Pulmonary arterial hypertension (PAH) is a multifaceted disease with poor prognosis and very high mortality rate.[1] PAH has sex bias and is more common in women than in men (in an approximate 3 : 1 ratio).[2,3] However, compared with men, women develop less severe hypertension and have a better 5-year survival rate.[3,4] In both sexes, pulmonary artery constriction, remodeling, and inflammation contribute to the pathogenesis of PAH.[2,3] Damage to the endothelium and endothelial dysfunction play critical roles in constricting the blood vessels and contribute to pulmonary artery remodeling, and both of these conditions increase pulmonary resistance and impair blood flow through the lungs leading to PAH.[5-8]

Endothelium-derived autacoids, like nitric oxide and eicosanoids, are well-known regulators of vascular function.[9] Nitric oxide and prostacyclin relax adjacent smooth-muscle cells and prevent smooth-muscle and endothelial cells from proliferating.[9,10] Reduction of nitric oxide and prostacyclin levels has been associated with the pathogenesis of PAH.[11,12] Therefore, therapies that increase nitric oxide or nitric oxide–dependent signaling and prostacyclin analogs are standards of treatment for PAH.[13]

Epoxyeicosatrienoic acids (EETs), hyperpolarizing factors, are produced by endothelial cytochrome P450 (CYP) epoxygenases.[14] EETs protect coronary and pulmonary artery endothelial cells from apoptosis, relax smooth-muscle cells, and dilate systemic arteries.[15] Paradoxically, EETs elicit contraction of rat[16] and rabbit[17] pulmonary arteries. However, their role in mediating pulmonary hypertension (PH) is controversial. In mice, EETs are generated from arachidonic acid by CYP2C9, CYP2C38, CYP2C39, CYP2C44, and CYP2J5.[18-20] The expression of CYP2C29 is increased in lungs of hypoxic mice, and hypoxia-induced PH is potentiated by ectopic expression of CYP2C9, a human homologue, in mice.[21] In contrast, monocrotaline-induced inflammation and PH in rats are ameliorated by inhibition of soluble epoxide hydrolase and overexpression of the *Cyp2j5* gene,[22,23] whereas deletion of the *Cyp2j* gene locus does not attenuate the hypoxia-elicited pulmonary artery remodeling and hypertension in male and female mice.[24] CYP2C29 and CYP2J5 are equally expressed in hepatic and extrahepatic tissues of both sexes, whereas CYP2C44 is expressed more in the kidneys and adrenal glands of female mice than male mice.[18-20] Since CYP2C44 shows a sex preference, we postulated that differential regulation of CYP2C44 expression and activity could play a role in mediating less severe PH in females versus males. To test this hypothesis, we exposed $Cyp2c44^{-/-}$ mice of both sexes to hypoxia for 5 weeks. Our

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results suggest that $Cyp2c44^{-/-}$ exacerbated PH and heart failure in female mice but not in male mice, and this was not mediated by decreased EETs but was potentially mediated by increased aldosterone.

METHODS

All experiments were performed following an institutional animal care and use committee–approved protocol in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Male and female *129SvJ* (weight: 25–30 g) wild-type (WT) and $Cyp2c44^{-/-}$ mice were used in the study.

PH induction in mice

Mice were exposed to normobaric hypoxia (10% O_2) in a ventilated chamber for 5 weeks. The normoxic control mice were kept in room air for all 5 weeks. At the end of the experiments, mice were sacrificed, and lungs and heart were harvested for biochemical and histological analyses.

Echocardiography

Echocardiography was performed in 2% isoflurane–anesthetized mice using a Vevo 770 imaging system (VisualSonics, Toronto, Ontario, Canada). In brief, at the beginning of the experiment (week 0) and at the end of the experiment (week 5), two-dimensional parasternal short-axis view was obtained, M-mode assessment of left ventricular function was performed, and left ventricular parameters were measured as described previously.[25] A two-dimensional parasternal short-axis view at the level of the aortic valve was obtained, and a pulsed-wave Doppler recording of the pulmonary artery blood flow was recorded as described elsewhere.[26] The ratio of pulmonary artery acceleration time (PAAT; time taken from start of flow to maximal velocity) to ejection time (ET; time taken from start of flow to the end of flow) was determined as described previously.[26,27] The PAAT/ET ratio is inversely related to pulmonary vascular resistance.

Hemodynamic

Hemodynamic measurements were performed as described elsewhere.[28] Briefly, at the end of the experiment protocol (week 5), the mice were anesthetized by 4% isoflurane, and 2% isoflurane was used to maintain anesthesia for the entire duration of the surgery and data acquisition. Body temperature of the animal during the surgery was maintained using a heating pad. Approximately 3 cm^2 of skin over the ventral neck region was exposed to locate and carefully isolate the right common carotid artery. A 1.4F Millar Micro-Tip pressure catheter was then inserted into the artery and advanced into the left ventricle for measurement of left ventricular hemodynamic parameters.

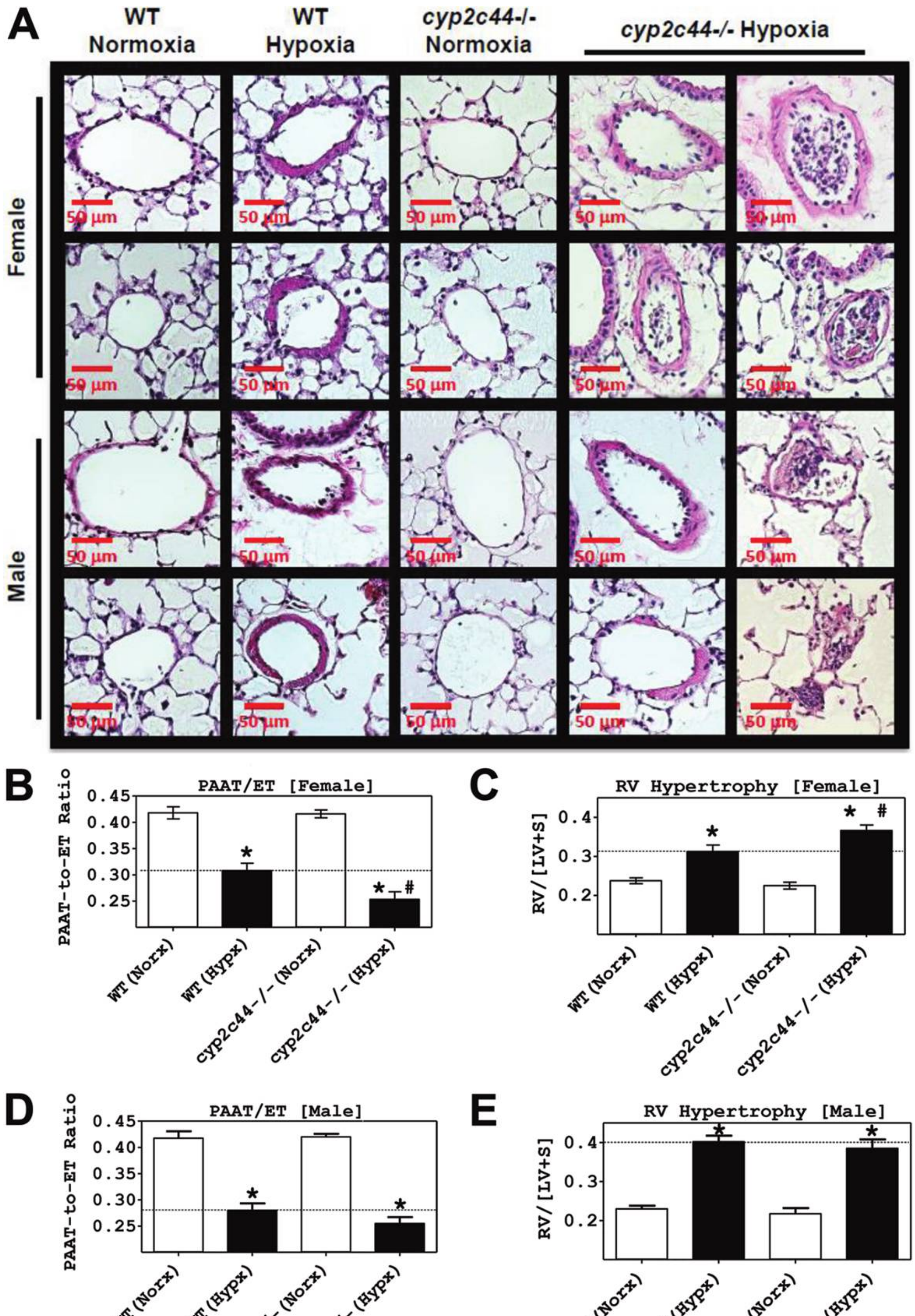
Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of EETs

For the mass spectrometry analysis, we used heart and lungs from $Cyp2c44^{-/-}$ and WT mice. Dissected lungs and hearts were then weighed and submerged in liquid nitrogen, and their lipids were extracted as described elsewhere.[29] Lipid extracts were subjected to alkaline hydrolysis, after which the eicosanoids present in lipid extracts were quantified by LC-MS/MS (Shimadzu Triple Quadrupole Mass Spectrometer LCMS-8050 equipped with a Nexera ultra high performance liquid chromatography (UHPLC) to monitor multiple reaction). Mass spectrometry conditions were as follows. Ionization mode: negative heated electrospray with applied voltage of −4.5 to approximately −3.0 kV; nebulizer gas: 3.0 L/min N_2; drying gas: 5.0 L/min N_2; heating gas: 12.0 L/min air; interface temperature: 400°C; desolvation lines temperature: 100°C; heat block temperature: 500°C; and internal standards: D6 20-hydroxyeicosatetraenoic acid (HETE), D8 5-HETE, D4 prostoglandin E2 (PGE_2), D11 11,12 DHET, D8 14(15) EET. UHPLC conditions were as follows. Analytical column: Zorbax Eclipse Plus C18 Rapid Resolution High Definition (50 mm L × 2.1 mm ID, 1.8 μm); mobile phase A: 95% water, 5% acetonitrile, 0.05% acetic acid; mobile phase B: acetonitrile 0.05%; time program: 40% B (0 minutes)→75% B (3 minutes)→85% B (7.5 minutes); flow rate: 0.4 mL/min; injection volume: 5 μL; and column oven temperature: 40°C. Multiple reaction monitoring transitions were as follows. 20-HETE, CE 19.5 m/z: 319.2→289.2; D6 20-HETE, CE 19.0 m/z: 325.2→295.2; 15-HETE, CE 13.0 m/z: 319.2→219.2; 12-HETE, CE 13.5 m/z: 319.2→179.1; 5-HETE, CE

Table 1. Epoxyeicosatrienoic and dihydroxyeicosatetraenoic acids in heart and lungs of wild-type versus *Cyp2c44* knockout ($Cyp2c44^{-/-}$) male and female mice

	Epoxyeicosatrienoic and dihydroxyeicosatetraenoic acids, pg/μg protein							
	Heart				Lungs			
	Male		Female		Male		Female	
Condition	Wild type	$Cyp2c44^{-/-}$	Wild type	$Cyp2c44^{-/-}$	Wild type	$Cyp2c44^{-/-}$	Wild type	$Cyp2c44^{-/-}$
Normoxia	273 ± 11	265 ± 23	99 ± 7	164 ± 40	119 ± 40	124 ± 17	104 ± 11	61 ± 6
Hypoxia	283 ± 19	369 ± 48	508 ± 203[a]	382 ± 83[a]	74 ± 10	193 ± 17	64 ± 10[a]	130 ± 15[a]

Note: Data are mean ± standard deviation.
[a] $P < 0.05$ versus normoxia; $N = 5$.


A
WT Normoxia
WT Hypoxia
cyp2c44-/- Normoxia
cyp2c44-/- Hypoxia
Female
Male
50 μm
B
PAAT/ET [Female]
PAAT-to-ET Ratio
0.45
0.40
0.35
0.30
0.25
WT (Norx)
WT (Hypx)
cyp2c44-/-(Norx)
cyp2c44-/-(Hypx)
C
RV Hypertrophy [Female]
RV/[LV+S]
0.4
0.3
0.2
D
PAAT/ET [Male]
E
RV Hypertrophy [Male]

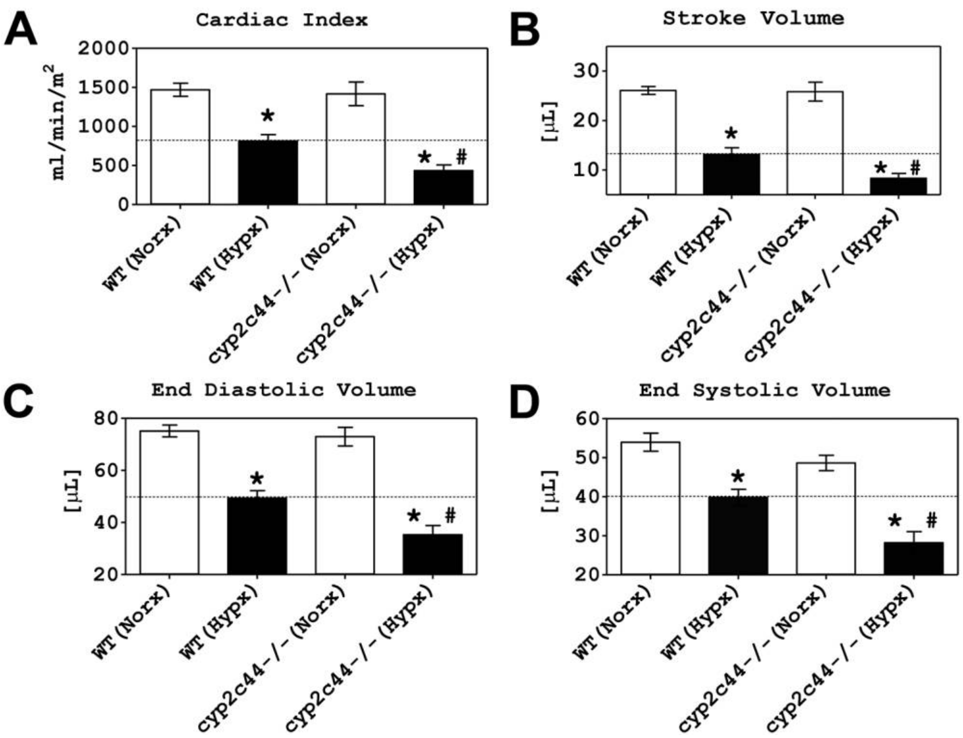


Figure 2. Effects of chronic hypoxia on cardiac hemodynamics in wild-type (WT) versus *Cyp2c44* knockout (*Cyp2c44*$^{-/-}$) female mice are shown. Cardiac index (*A*), stroke volume (*B*), end-diastolic volume (*C*), and end-systolic volume (*D*) were decreased in hypoxic (Hypx) WT and *Cyp2c44*$^{-/-}$ groups. However, deletion of *Cyp2c44* gene exacerbated heart failure. $N = 5$ in each group. Asterisk indicates $P < 0.05$ versus normoxic (Norx) controls, and pound sign indicates $P < 0.05$ versus Hypx WT.

15.0 m/z: 319.2→115.0; D8 5-HETE, CE 14.5 m/z: 327.5→116.0; PGE$_2$, CE 16.0 m/z: 351.1→271.2; D4 PGE$_2$, CE 18.0 m/z: 355.3→275.2; D11 11,12 DHET, CE 21.0 m/z: 348.5→167.1; 14,15 DHET, CE 18.5 m/z: 337.3→207.1, 11,12 DHET, CE 19.5 m/z: 337.3→167.1; 8,9 DHET, CE 22.5 m/z: 337.3→145.0; 5,6 DHET, CE 18.0 m/z: 337.3→145.0; D8 14(15) EET, CE 13.5 m/z: 330.3→218.9; 14(15) EET, CE 11.5 m/z: 319.2→219.2; 11(12) EET, CE 14.5 m/z: 319.2→167.2; 8(9) EET, CE 16.5 m/z: 319.2→126.9; and 5(6) EET, CE 319.2 m/z: 319.2→191.3.

Histology

Mice were euthanized and lungs and heart were harvested for molecular biological, biochemical, and histological analyses. The left lung lobe was inflated with 0.5% agarose in 1% neutral buffered formalin at 20 cm H_2O pressure and fixed in 10% neutral buffered formalin overnight.[30] Formalin-fixed lung was blocked and embedded in paraffin. Formalin-fixed, paraffin-embedded sections were cut at 5 μm for the immunohistological analysis in the core histology laboratory at New York Medical College. The inferior and post caval lobes of the right lung were snap frozen for biochemical analysis. Hearts were fixed in formalin, and the right ventricle (RV) and left ventricle plus septum (LV+S) were weighed for calculation of RV/LV+S and used for hematoxylin and eosin staining.

Aldosterone assay

Aldosterone levels were measured in plasma samples obtained from the *Cyp2c44*$^{-/-}$ and WT mice using an enzyme-linked immunosorbent assay kit (Abcam ab136933; Aldosterone ELISA Kit) according to the manufacturer's protocol.

Figure 1. Effect of chronic hypoxia on lung and heart remodeling and pulmonary vascular resistance in wild-type (WT) and *Cyp2c44* knockout (*Cyp2c44*$^{-/-}$) female and male mice. *A*, Representative micrographs of lung sections from normoxic (Norx) and hypoxic (Hypx) WT and *Cyp2c44*$^{-/-}$ females and males stained with hematoxylin and eosin show medial thickening of arterial wall, perivascular cuffing, and blood cells/thrombus in pulmonary vessels of hypoxic knockout mice. *B*, Summary data of pulmonary artery acceleration time to ejection time ratio (PAAT/ET) ratio. *C*, Right ventricle (RV) hypertrophy determined by Fulton index in WT and *Cyp2c44*$^{-/-}$ group is demonstrated. $N = 5$ in each group. Asterisk indicates $P < 0.05$ versus normoxia (Nor) controls, and pound sign indicates $P < 0.05$ versus WT hypoxia. Magnification ×20. LV+S: left ventricle plus septum.

Statistical analysis

Values are mean ± SEM of the number of samples (n) from different animals. Statistical analyses were performed with unpaired Student t test, and a one-way ANOVA with Bonferroni correction was used for comparing multiple groups. $P < 0.05$ was used to establish statistical significance.

RESULTS

EETs synthesis in heart and lungs was not reduced by *Cyp2c44* gene disruption

All (14,15; 11,12; 8,9; 5,6) EETs and DHETs were quantitated in the heart and lungs of control and hypoxic PH mice. Total EETs (EETs+DHETs) in heart and lung tissues of male and female mice were not reduced by *Cyp2c44* gene ablation (Table 1). Interestingly, total EETs were increased in the heart and lung tissues of chronically hypoxic WT and *Cyp2c44*$^{-/-}$ females but not in males.

Pulmonary vascular remodeling, pulmonary vascular resistance, and right ventricle hypertrophy are exacerbated by *Cyp2c44* gene deletion in female mice

WT and *Cyp2c44*$^{-/-}$ female and male mice exposed to hypoxia for 5 weeks developed PH, compared with their normoxic controls. Chronic hypoxia elicited pulmonary vascular remodeling, defined as medial wall thickening of large arteries, in both WT and *Cyp2c44*$^{-/-}$ female and male mice (Fig. 1*A*). In addition, we observed marked perivascular cuffing and thrombosis and occluded pulmonary vessels in *Cyp2c44*$^{-/-}$ hypoxia females and males but not in WT normoxia females or males (Fig. 1*A*). We also found that the PAAT/ET ratio, and RV hypertrophy (RV/LV+S) were, respectively, decreased (Fig. 1*B*) and increased (Fig. 1*C*) in the hypertensive *Cyp2c44*$^{-/-}$ females as compared with WT females. Interestingly, both the PAAT/ET ratio and RV hypertrophy were, respectively, decreased and increased to the same degree in hypertensive *Cyp2c44*$^{-/-}$ and WT males (Fig. 1*D* and 1*E*).

Heart function deteriorated more in *Cyp2c44*$^{-/-}$ females exposed to chronic hypoxia

The heart function was determined by inserting a pressure-volume Millar catheter into the LV. Cardiac index (CI; Fig. 2*A*), stroke volume (SV; Fig. 2*B*), end-diastolic volume (EDV; Fig. 2*C*), and end-systolic volume (ESV; Fig. 2*D*) were decreased more in hypertensive *Cyp2c44*$^{-/-}$ than in WT female mice. In male mice, we did not find any differences in hemodynamic parameters between the hypertensive *Cyp2c44*$^{-/-}$ and WT groups (Table 2).

LV stiffness and systemic arterial elastance are increased in *Cyp2c44*$^{-/-}$ females exposed to chronic hypoxia

LV stiffness, calculated from the ratio of dP/dt_{max} to dV/dt_{max}, is increased more in hypertensive *Cyp2c44*$^{-/-}$ females than in hypertensive WT and *Cyp2c44*$^{-/-}$ controls (Fig. 3*A*), but no differences in trichrome staining of the LV were noted among these groups (Fig. 3*B*). In addition, systemic arterial elastance (Fig. 3*C*), a measure of large artery stiffness, and total peripheral vascular resistance (Fig. 3*D*), calculated from dividing cardiac output by mean systemic arterial pressure, were higher in hypertensive *Cyp2c44*$^{-/-}$ females than in hypertensive WT and *Cyp2c44*$^{-/-}$ controls. In male mice, the increases in LV stiffness and arterial elastance were not different between the chronically hypoxic *Cyp2c44*$^{-/-}$ and WT mice (Table 2).

Aldosterone levels are elevated in *Cyp2c44*$^{-/-}$ female mice exposed to chronic hypoxia

Aldosterone plays a critical role in the homeostasis of blood volume and pressure[31] as well as in heart and blood vessel remodel-

Table 2. Hemodynamic changes in pulmonary normotensive versus hypertensive male wild-type and *Cyp2c44* knockout (*Cyp2c44*$^{-/-}$) mice

	Wild type		*Cyp2c44*$^{-/-}$	
	Control	Chronic hypoxic pulmonary hypertension	Control	Chronic hypoxic pulmonary hypertension
CI (mL/min/m^2)	1,302 ± 89.38	308.1 ± 19.03[a]	1,344 ± 114.8	304.6 ± 36.01[a]
SV (μL)	29.83 ± 1.50	5.90 ± 0.91[a]	28.50 ± 2.09	6.03 ± 0.50
EDV (μL)	84.78 ± 2.28	26.31 ± 2.56[a]	72.96 ± 3.59	29.00 ± 2.59[a]
ESV (μL)	60.20 ± 2.71	21.62 ± 1.87[a]	48.64 ± 2.45	25.21 ± 2.39[a]
LV stiffness (mmHg/μL)	4.31 ± 0.17	21.89 ± 4.91[a]	4.43 ± 0.15	18.26 ± 3.85[a]
Ea (mmHg/μL)	3.57 ± 0.13	14.63 ± 2.22[a]	3.10 ± 0.22	14.49 ± 1.82[a]
TPR (mmHg × min/mL)	7.58 ± 0.38	34.51 ± 4.38[a]	6.92 ± 0.55	34.20 ± 2.90[a]

Note: Data are mean ± standard deviation. CI: cardiac index; Ea: arterial elastance; EDV: end-diastolic volume; ESV: end-systolic volume; LV: left ventricle; SV: stroke volume; TPR: total peripheral resistance.

[a] $P < 0.05$ versus control; $N = 5$.

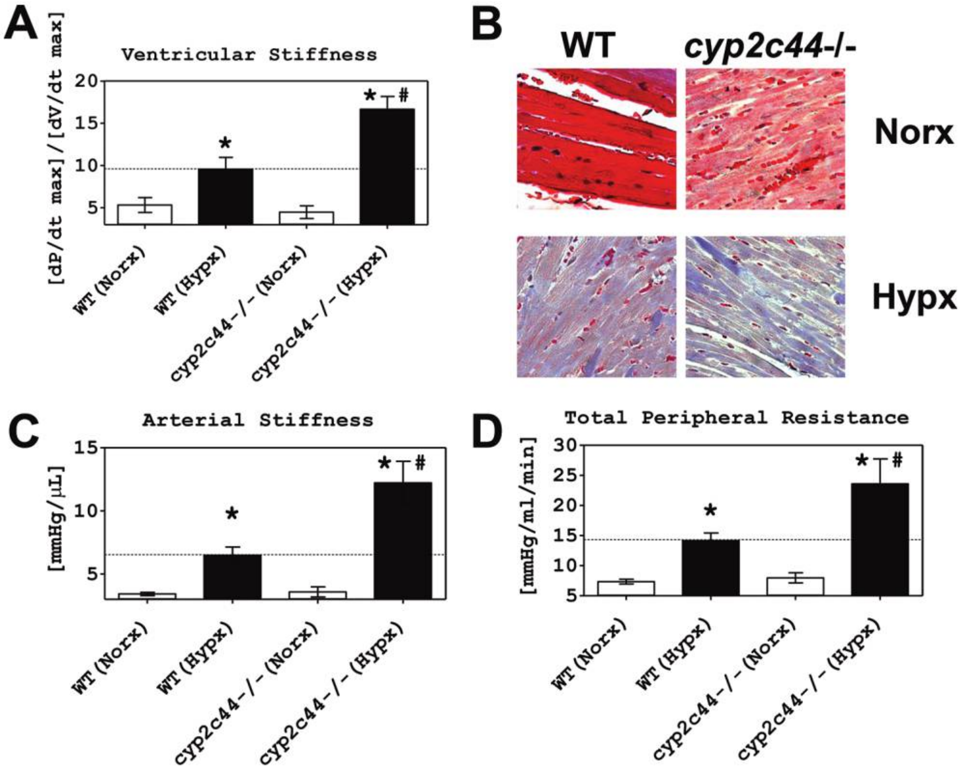


Figure 3. Effects of chronic hypoxia on left ventricle and arterial stiffness in wild-type (WT) versus *Cyp2c44* knockout ($Cyp2c44^{-/-}$) female mice are shown. Left ventricle stiffness (*A*), trichrome staining of left ventricle free wall (fibrosis; *B*), systemic arterial elastance (*C*), and total peripheral vascular resistance (*D*) were increased in both hypoxic (Hypx) WT and $Cyp2c44^{-/-}$ groups. However, deletion of the *Cyp2c44* gene exacerbated ventricle and arterial stiffness and further increased total peripheral resistance. $N = 5$ in each group. Asterisk indicates $P < 0.05$ versus normoxia (Norx) controls, and pound sign indicates $P < 0.05$ versus Hypx WT.

ing in animals and humans.[32] Earlier studies have shown that high plasma aldosterone levels correlate well with remodeling of the heart, but more so in women than in men.[33] Because angiotensin II and blood levels of Na^+ and K^+ regulate synthesis of aldosterone, we estimated aldosterone in plasma samples obtained from control and PH WT and $Cyp2c44^{-/-}$ female and male mice. Aldosterone levels in females were decreased in PH versus control WT mice, but they were increased in PH versus control $Cyp2c44^{-/-}$ mice (Fig. 4). This increase in circulating aldosterone levels was not seen in the male PH versus control $Cyp2c44^{-/-}$ mice (Table 3).

DISCUSSION

It is well known that PAH has a sex bias and that the disease is more prevalent among women than men.[2-4] In contrast, in several animal models of PH, the severity of the hypertension is less in females than in males.[34,35] In this study of mice, we found that disruption of the *Cyp2c44* gene worsened the severity of PH in females exposed to hypoxia, thus minimizing the apparent advantage in their response to hypoxia. We also found that the exacerbated hypoxia-induced pulmonary vascular remodeling and heart failure associated with disruption of the *Cyp2c44* gene in female mice were not due to a reduction in pulmonary or heart EETs but were potentially linked to increases in the levels of circulating aldosterone.

The CYP family of enzymes expressed in hepatic and extrahepatic tissues metabolizes steroids, fatty acids, and drugs and has diverse actions on cell and organ function.[18,36] Overexpression of the estrogen-metabolizing enzyme CYP1B1 in pulmonary arteries has been implicated in the development of PH in mice, rats, and humans.[3,37-39] Estrogen-induced *Cyp2c29* gene expression in female mice increases vascular EET synthesis and blood vessel dilation in the absence of nitric oxide.[40] In contrast to these observations, CYP2C29 expression is increased in the lungs of mice exposed to hypoxia for 2 hours, and hypoxia-induced PH and pulmonary vascular remodeling appear to be attenuated by continuous treatment of mice with the expoxygenase inhibitor N-methylsufonyl-6-[2-propargyloxyphenyl] hexanamide.[21] Moreover, it was found that overexpression of CYP2C9, a human epoxygenase homologue, in mice elevates mean pulmonary arterial pressure and total pulmonary vascular resistance and that disruption, but not inhibition, of soluble epoxide hydrolase contributes to the pathophysiology of hypoxia-induced pulmonary artery remodeling and PH.[21,41] In contrast, other studies have reported that inhibition of soluble epoxide hydrolase[22] or overexpression of CYP2J5[23] reduce monocrotaline-induced pulmonary vascular remodeling and PH in mice. Although it was recently found that *Cyp2j* deficiency does not attenuate hypoxia-induced PH in male and female mice, we now demonstrate that knockout of CYP2C44 epoxygenase, which metabolizes arachidonic

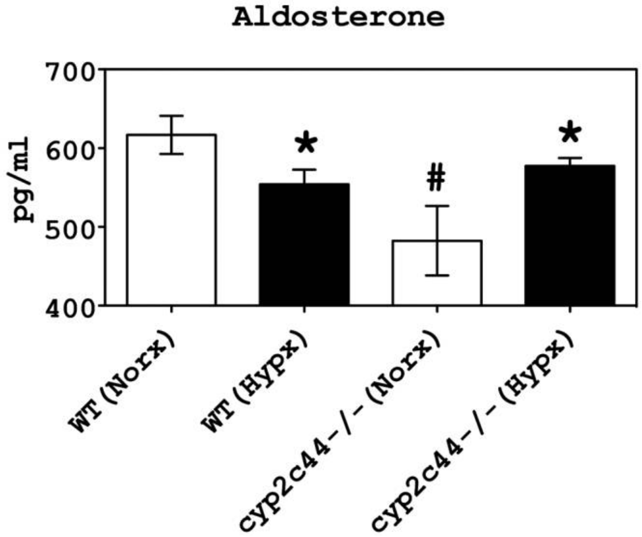


Figure 4. Effect of chronic hypoxia on circulating aldosterone levels in wild-type (WT) versus *Cyp2c44* knockout ($Cyp2c44^{-/-}$) female mice is shown. Plasma aldosterone was increased in pulmonary hypertensive $Cyp2c44^{-/-}$ as compared with normotensive control and hypertensive WT group. Four mice were measured in duplicate in each group. Asterisk indicates $P < 0.05$ versus normoxia (Norx) controls, and pound sign indicates $P < 0.05$ versus Norx WT. Hypx: hypoxic.

acid predominantly to EETs, increases the severity of PH and heart failure in female but not male mice.[24]

CYP2C44 has little homology with other mouse CYP2C expoxygenases but is highly homologous to the rat kidney CYP2C23.[19] CYP2C44 is predominantly expressed in liver, kidneys, and adrenal glands.[19] Moreover, expression of CYP2C44 messenger RNA in kidneys and adrenal glands is 2-fold higher among females than males. Our results indicate that deletion of the *Cyp2c44* gene exacerbated PH-associated heart failure in females. Furthermore, perivascular cuffing, an indication of edema, and the hemodynamic outcome were more severe in $Cyp2c44^{-/-}$ females than in WT females exposed to hypoxia for 5 weeks. This suggests that deletion of *Cyp2c44* gene exacerbated pulmonary vascular remodeling, RV hypertrophy, LV and arterial stiffening, and reduction of CI.

CYP-derived EETs are hyperpolarizing factors that decrease the membrane potential of vascular smooth-muscle cells and dilate renal arteries and other systemic blood vessels.[14,42] Paradoxically, EETs constrict pressurized rabbit pulmonary arteries and increase pulmonary arterial pressure.[17,21,43] In isolated perfused human lungs, it was observed that CYP-derived *cis*-EETs released into the vascular space by A23187 and inflammatory challenge correlated with the vasopressor response.[44] In this study, we observed that *Cyp2c44* deletion did not significantly reduce EET levels in heart or lungs of either sex. Instead, EETs increased in heart and lungs of $Cyp2c44^{-/-}$ PH females but not males. This indicates that the synthesis of EETs by CYP2C29 or other CYP epoxygenases expressed in lungs and heart was increased in hypertensive females. Alternatively, circulating EETs through a paracrine action, like hormones or cytokines, stimulate EET-associated receptor signaling in lungs and heart to exacerbate PH in females. Nonetheless, we did not find a correlation between EET levels and the pulmonary vascular resistance or RV hypertrophy in males and females. All together, these results suggest that EETs do not contribute significantly to the development of hypoxia-induced PH or heart failure in mice.

On the basis of the lack of evidence for lung- or heart-derived EETs in the pathogenesis of PH-associated heart failure, we speculated that some factor other than CYP2C44-derived EETs played a potential role in promoting pulmonary vascular remodeling and suppressing cardiac function. CYP2C44 is expressed in the kidney, and reduction of EETs resulting from the deletion of the *Cyp2c44* gene increases the activity of epithelial Na^+ channel (ENaC) in the cortical collecting duct, which facilitates Na^+ and K^+ reabsorption and modulates blood levels of these ions.[19,45-48] Blood K^+ levels are critical determinants of the production of aldosterone, a mineralocorticoid hormone produced by the adrenal gland that increases blood volume and blood pressure.[49] To the best of our knowledge, there is no evidence that CYP2C44 has a direct influence on the biosynthesis of aldosterone. Therefore, we postulated that an increase of ENaC activity by disruption of CYP2C44 could regulate circulating aldosterone levels. Our findings indicated that, in normoxic control mice, aldosterone levels were lower in $Cyp2c44^{-/-}$ females than in WT females. Furthermore, although aldosterone levels were slightly but significantly decreased in WT mice, paradoxically, aldosterone increased in $Cyp2c44^{-/-}$ female mice exposed to hypoxia for 5 weeks compared with their normoxic controls. Concomitantly, hypoxia-induced PH and heart failure were exacerbated in $Cyp2c44^{-/-}$ female mice when compared with WT female mice. Thus, these results suggest that disruption of the *Cyp2c44* gene has a consequence on aldosterone levels in the chronically hypoxic females. Increased circulating levels of aldosterone are deleterious to cardiovascular function and provoke pathogenic remodeling of the cardiovascular system, whereas aldosterone levels correlate well with remodeling of the heart, although more so in females than in males.[32,33] There is also an association between elevated plasma aldosterone and Na^+ levels with increased incidence of coagulation and thrombotic events, which are mediated by aldosterone-induced AT1R signaling and blocked by mineralocorticoid receptor antagonists.[50-53] Along these line, thrombosis and occluded pulmonary ar-

Table 3. Circulating aldosterone levels in wild-type versus *Cyp2c44* knockout ($Cyp2c44^{-/-}$) male mice

	Circulating aldosterone, pg/mL	
Condition	Wild type	$Cyp2c44^{-/-}$
Normoxia	543 ± 35	546 ± 11
Hypoxia	611 ± 7[a]	556 ± 15

[a] $P < 0.05$ versus normoxia; $N = 5$.

teries were apparent in hypoxic $Cyp2c44^{-/-}$ female mice but not WT female mice. Thrombosis will increase pulmonary vascular resistance, and thrombin-activated platelets will release platelet-derived growth factor that will stimulate pulmonary artery remodeling. A recent study found that aldosterone levels are elevated in patients with idiopathic PAH, and the steroid has been proposed as a potential biomarker.[54] Furthermore, activation of the mineralocorticoid receptor contributes to pulmonary vascular and right ventricular remodeling of hypoxia- and monocrotaline-induced PH in mice and rats, respectively.[55] Therefore, we imply increased aldosterone as one of the causes for the development of more severe hypoxia-induced pulmonary vascular remodeling, RV hypertrophy, and LV and systemic artery stiffness in $Cyp2c44^{-/-}$ female mice.

In summary, we have demonstrated that hypoxia-induced pulmonary vascular remodeling and cardiac dysfunction are amplified by *Cyp2c44* gene deletion in female, but not male, mice. This raises the possibility that CYP2C44 could play a role in protecting females from PH and could be one of the underlying causes for a better 5-year survival rate among women than among men.

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