

# Soluble epoxide hydrolase plays an essential role in angiotensin II-induced cardiac hypertrophy

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Pathophysiological cardiac hypertrophy is one of the most common causes of heart failure. Epoxyeicosatrienoic acids, hydrolyzed and degraded by soluble epoxide hydrolase (sEH), can function as endothelium-derived hyperpolarizing factors to induce dilation of coronary arteries and thus are cardioprotective. In this study, we investigated the role of sEH in two rodent models of angiotensin II (Ang II)-induced cardiac hypertrophy. The protein level of sEH was elevated in the heart of both spontaneously hypertensive rats and Ang II-infused Wistar rats. Blocking the Ang II type 1 receptor with losartan could abolish this induction. Administration of a potent sEH inhibitor (sEHI) prevented the pathogenesis of the Ang II-induced hypertrophy, as demonstrated by decreased left-ventricular hypertrophy assessed by echocardiography, reduced cardiomyocyte size, and attenuated expression of hypertrophy markers, including atrial natriuretic factor and  $\beta$ -myosin heavy chain. Because sEH elevation was not observed in exercise- or norepinephrine-induced hypertrophy, the sEH induction was closely associated with Ang II-induced hypertrophy. *In vitro*, Ang II upregulated sEH and hypertrophy markers in neonatal cardiomyocytes isolated from rat and mouse. Expression of these marker genes was elevated with adenovirus-mediated sEH overexpression but decreased with sEHI treatment. These results were supported by studies in neonatal cardiomyocytes from sEH<sup>-/-</sup> mice. Our results suggest that sEH is specifically upregulated by Ang II, which directly mediates Ang II-induced cardiac hypertrophy. Thus, pharmacological inhibition of sEH would be a useful approach to prevent and treat Ang II-induced cardiac hypertrophy.

epoxyeicosatrienoic acid | cardiomyocyte | activator protein 1

Cardiac hypertrophy is a major pathological event leading to heart failure (1). Both animal models and human diseases show hypertrophic impairments associated with an increased induction of the fetal gene program and repression of some adult cardiac genes (2). This pathophysiological condition is characterized by increased cardiomyocyte size, augmented protein synthesis, and altered gene expression. An increase in wall tension resulting from prolonged cardiac hypertrophy switches the myosin heavy chain (MHC) from the  $\alpha$ - to the  $\beta$ -isoform and increases the expression of atrial natriuretic factor (ANF). Related to compensatory attenuation of wall stress, this change in gene expression is manifested in pathological cardiac hypertrophy (3, 4), the leading cause of cardiac remodeling and heart failure (5).

Arachidonic acid, derived from membrane phospholipids, can be converted to eicosanoids via three major enzymatic pathways, namely, cyclooxygenase, lipoxygenase, and CYP 450 epoxygenase. In addition to generating  $\omega$ - and  $\omega$ -1 hydroxylation products, CYP 450 epoxygenase produces 4 epoxyeicosatrienoic acid (EET) regioisomer metabolites: 5,6-, 8,9-, 11,12-, and 14,15-EET (6). EETs exert membrane potential-independent effects and modulate several signaling cascades that affect cell function by increasing intracellular Ca<sup>2+</sup> concentration and activating a

large conductance of the Ca<sup>2+</sup>-activated K<sup>+</sup> channel. EETs also cause vasodilation, which suggests that they contribute to the action of the endothelium-derived hyperpolarizing factor (6).

Soluble epoxide hydrolase (sEH, Ephx2) catalyzes the conversion of EETs to the corresponding dihydroxyeicosatrienoic acids (DHETs) (7, 8). An early study demonstrated that a selective sEH inhibitor reversed the hypertensive phenotype of the spontaneously hypertensive rat (SHR) (7). Angiotensin II (Ang II), a potent vessel constrictor, elevates blood pressure by acting on several tissue types. I.p. injection of sEH-selective inhibitors in Ang II-infused hypertensive rats greatly increased the level of EETs and lowered systolic blood pressure (9). Thus, increased production of EETs by CYP 450s or decreased hydrolysis by sEH inhibition seems to reduce blood pressure *in vivo*. A preponderance of evidence demonstrates that sEH is also involved in cardiac hypertrophy. Recent studies of pressure overload-induced hypertrophy in mice showed that sEH inhibition prevented cardiac enlargement (10). Thus, the enzyme activity of sEH appears to be involved in the pathogenesis of cardiac hypertrophy and hypertension.

Previous work indicated that the use of sEH inhibitors to increase the level of lipid epoxides may reduce cardiac hypertrophy by blocking NF- $\kappa$ B activation (10). We also found that activator protein 1 (AP-1) is involved in the transcriptional upregulation of sEH by Ang II in vascular endothelial cells (ECs), which may contribute to Ang II-induced hypertension (11). Given the potential role of sEH in the Ang II-induced cardiac hypertrophy, we studied whether sEH is upregulated in the hypertrophic heart of the SHR and the Ang II-infused rat. Our results showed a specific upregulation of sEH by Ang II, which is necessary and sufficient for the induction of cardiac hypertrophy. Moreover, a newly developed sEH inhibitor, 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS), could largely attenuate the Ang II-induced cardiac hypertrophy.

## Results

**Ang II Upregulates sEH in the Hypertrophic Heart.** To investigate the role of sEH in Ang II-induced cardiac hypertrophy, we used rat

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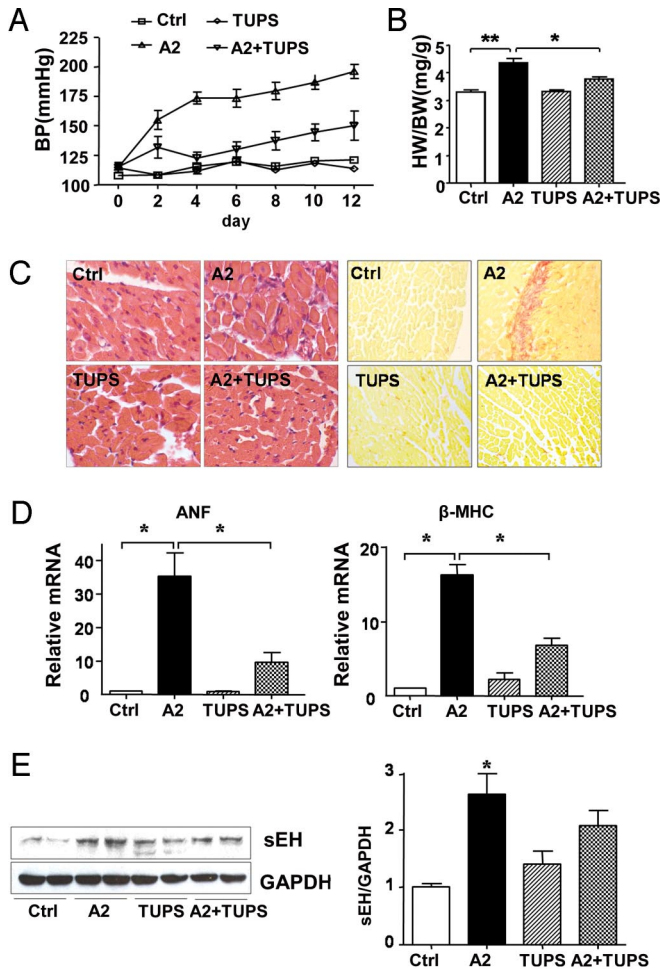
Conflict of interest statement: P.D.J., N.C., and B.D.H. authored University of California patents in this area. B. D. Hammock founded Arete Therapeutics to evaluate inhibitors of the soluble epoxide hydrolase as therapeutic agents. Arete has licensed UC IP in this area. None of the authors received funding for this work from Arete Therapeutics, and Arete Therapeutics scientists did not contribute to these studies.

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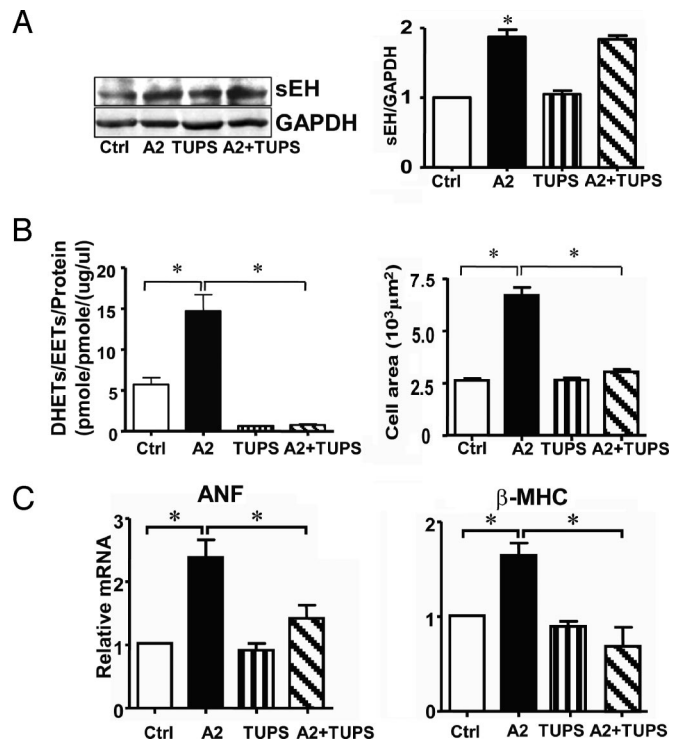




**Fig. 3.** sEH inhibitor attenuates Ang II-induced cardiac hypertrophy in Sprague–Dawley rats. Sprague–Dawley rats (180–280 g, male) received TUPS (0.65 mg/kg/day) or PEG400 by oral gavage for 21 days. Seven days after beginning TUPS administration, a minipump was implanted in the dorsal region to deliver Ang II (A2) for 14 days. PBS treatment was used as a control (Ctrl). (A) Systolic blood pressure (BP) was measured every 2 days after implantation. (B) Heart weight to body weight (HW/BW) was measured after rats were killed. (C) Cross-sections of rat left ventricles histochemically stained with picric-sirius red for fibrosis. (D) Real-time RT–PCR analysis of ANF and  $\beta$ -MHC mRNA level. Data are means  $\pm$  SD of the relative mRNA normalized to that of 18S from 6 rats in each group. (E) Western blot analysis of sEH and GAPDH proteins was performed. The results are presented as mean  $\pm$  SD from 6 rats in each group (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

(exercise) or pathophysiological (NE) cardiac hypertrophy we tested.

**sEH Inhibition Blocks Ang II-Induced Cardiac Hypertrophy in Vivo and in Vitro.** To decipher whether the Ang II-induced hypertrophy was due to sEH upregulation, we infused Sprague–Dawley rats without or with Ang II (450 ng/kg/min) for 14 days. Half of the rats from each group were given the sEH inhibitor TUPS (0.65 mg/kg/day) or polyethylene glycol 400 (PEG400) as a control. Systolic blood pressure (SBP) in control rats and those treated with TUPS alone remained within the normotensive range throughout the experimental period, but Ang II administration resulted in severe hypertension with a large increase in ratio of heart weight to body weight (Fig. 3A and B). Importantly, the hypertension and cardiac hypertrophy were significantly attenuated in the TUPS-treated hypertensive rats. Echocardiography performed at the end of experiment revealed increased thickness

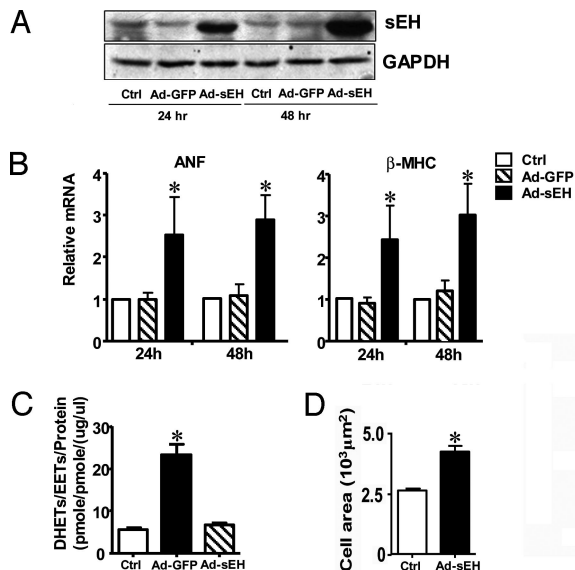


**Fig. 4.** Role of sEH in Ang II-induced cardiomyocyte hypertrophy: rat (A and C) or mouse (B). NCMs were treated with 100 nM Ang II (A2) with or without TUPS (1  $\mu$ M) for 24 h. (A) Western blot analysis was performed with GAPDH level as a control. (B) The DHETs and EETs in cytosolic extracts were separated on reverse-phase HPLC and then quantified by use of a 4000 QTRAP tandem mass spectrometer. For cell area, cells were stained with  $\alpha$ -actin, and the cell area of myocytes was measured on confocal microscopy. Data are means  $\pm$  SD of 100 cell measurements from 3 independent experiments. (C) Quantitative RT–PCR analysis of mRNA level of ANF and  $\beta$ -MHC, the relative mRNA normalized to that of 18S (\*,  $P < 0.05$ ).

of the left-ventricular wall at end systole/diastole, another indication of cardiac hypertrophy, in Ang II-infused rats; the cardiac hypertrophy was significantly reduced with sEH activity inhibited by TUPS (Fig. S3). Ejection fraction, a surrogate of systolic function, was increased in rats with Ang II infusion alone, which suggests compensatory responses. This effect was attenuated with TUPS treatment as well. The cell size and fibrosis seen in histological sections of the left ventricle from rats with Ang II infusion alone were greater than those in control specimens, which confirmed the heart hypertrophy and remodeling, but were attenuated with TUPS treatment (Fig. 3C). Further, the mRNA levels of ANF and  $\beta$ -MHC were higher in extracts isolated from hearts with Ang II infusion alone but lowered with TUPS treatment (Fig. 3D). The level of sEH protein was increased in the Ang II infusion group, which was not significantly decreased by TUPS (Fig. 3E). TUPS administration had no effect on the nonhypertrophic untreated group, which indicates that sEH inhibition had little effect on the endogenous renin–angiotensin system.

To define the role of sEH in hypertrophic cardiomyocytes, we applied TUPS to cultured NCMs. Although the sEH expression was unaffected (Fig. 4A), TUPS (1  $\mu$ M) markedly inhibited the basal and Ang II-induced sEH activity and cellular size of cardiomyocytes (Fig. 4B and Fig. S3). Consistent with results from in vivo experiments, the increase in ANF and  $\beta$ -MHC mRNA in cultured cells induced by Ang II was blocked by TUPS treatment (Fig. 4C).

**Direct Effect of Gene Manipulation of sEH on Hypertrophy in Cultured Cardiomyocytes.** We next studied whether adenoviral overexpression of sEH in cultured NCMs to mimic the induction by Ang II



**Fig. 5.** Role of sEH overexpression in cultured NCMs. (A and B) Rat NCMs were infected with Ad-sEH or Ad-GFP for 24 or 48 h. Western blot analysis of sEH protein level in A and quantitative RT-PCR analysis of mRNA level of ANF and  $\beta$ -MHC in B were measured. (C and D) Murine NCMs were infected with Ad-sEH or Ad-GFP for 24 h and sEH activity and the size of the myocytes were determined as described in Fig. 4. Data are means  $\pm$  SD from 3 independent experiments (\*,  $P < 0.05$ ).

would induce cardiac hypertrophy. The protein level and activity of sEH were increased significantly in adenovirus (Ad)-sEH-infected cells as compared with untreated or Ad-GFP-infected controls (Fig. 5A and C and Fig. S4). Of note, the cell area and expression of hypertrophic markers were increased in cells overexpressing sEH (Fig. 5B and D). In contrast, Ang II-induced cellular responses in wild-type cells were attenuated in cardiomyocytes isolated from Ephx2 gene-disrupted mice (12), as shown in Fig. 6. These results suggest that sEH not only functions as a mediator for the hypertrophic effect of Ang II but also is sufficient for inducing cardiac hypertrophy.

**Discussion**

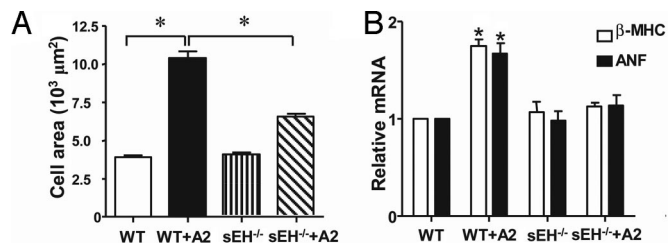
sEH inhibition with the ensuing decrease in EET degradation has been suggested to lower blood pressure in several models of hypertension, including one induced by Ang II (9, 13, 14). Administration of sEH inhibitors also attenuates cardiohypertrophy in murine models with transverse aortic constriction (TAC) (10). However, the involvement of sEH in Ang II-induced cardiac hypertrophy is unclear. In the current study, we found sEH upregulated in the heart of SHR and Ang II-infused Wistar

rats. The sEH upregulation by Ang II could be blocked by losartan, a blocker of the AT<sub>1</sub> receptor. Moreover, sEH upregulation occurred with Ang II-induced hypertrophy but not with NE-induced hypertrophy or that induced after swim training.

At the molecular level, Ang II induces ANF expression through the extracellular signal-related kinase (ERK) cascade in cultured neonatal rat ventricular myocytes (15). A catalytically inactive mutant of ERK has been shown to inhibit the Ang II-induced  $\beta$ -MHC promoter activity (16). Ang II-induced hypertension, cardiac hypertrophy, and reactive oxygen species production are possibly mediated by p38 mitogen-activated protein kinase (MAPK) (17, 18). Ang II can also activate Jun N-terminal kinase (JNK), which leads to the activation of the transcription factor AP-1 in vitro and in vivo (19–21). ERK, p38, and JNK constitute the MAPK family signaling that activates the downstream AP-1 transcription factor (22, 23). AP-1/DNA interaction was prevented by the AT<sub>1</sub> receptor antagonist in transgenic rats harboring both renin and angiotensinogen genes (24). Our previous work indicated that AP-1 activation is involved in the transcriptional upregulation of sEH by Ang II in ECs (11). We previously reported that basal promoter activity of sEH varied in different types of cells, of which a proximal Sp1 binding site was critical (25). During the pathophysiological onset of hypertension, Ang II likely induces sEH via AP-1 activation in adult ventricular cardiomyocytes (11). In the current study, we found that the AP-1 site in the promoter region was crucial for the Ang II activation of sEH promoter activity, since the mutation of the AP-1 site at -446 inhibited Ang II-induced promoter activities (Fig. S1D). However, as compared with the induction of protein and mRNA levels, luciferase activity driven by the sEH promoter was not markedly increased, which suggests the involvement of other factors, including other transcriptional regulation and possible posttranscriptional and posttranslational modification.

The elevated level of sEH would increase the hydrolysis of EETs and other epoxy lipids, but the administration of the sEH inhibitor TUPS should offset the increased sEH expression. We found that in vivo administration of TUPS prevented the Ang II-induced enlargement of the heart (Fig. 3). Because the enzyme activity of sEH was greatly reduced in cardiomyocytes (Fig. 4), we hypothesize TUPS exerts its cardioprotective effect, at least in part, through the increased EET activity. ATP-sensitive potassium channels ( $K_{ATP}$ ) have been suggested to be important for cardiac protection (26). The protective mechanisms contributed by these channels include depolarizing intramitochondrial membrane, altering reactive oxygen species production, and increasing mitochondrial  $K^+$  uptake with attendant reduction of  $Ca^{2+}$  overload (27–32). Although Ang II inhibits  $K_{ATP}$  activity in ventricular myocytes (33), EETs can increase the opening for both sarcolemmal and mitochondrial  $K_{ATP}$  (sarc  $K_{ATP}$  and mito  $K_{ATP}$ ). Cytochrome P450 CYP 2J2 overexpression in cardiomyocytes, presumably augmenting EET production, increases flavoprotein fluorescence, which is indicative of enhanced mito  $K_{ATP}$  activity (34). Indeed, treating cardiomyocytes with a physiological level of EETs increased flavoprotein fluorescence (35). Through inhibiting sEH, TUPS treatment thus is likely to increase the cardiac level of EETs and other epoxy lipids, which prevents the  $K_{ATP}$  inhibition by Ang II. Because MAPKs are involved in the upregulation of ANF and  $\beta$ -MHC, the increased EET activity may also inhibit  $K_{ATP}$  and MAPK to abolish the induction of these hypertrophic markers.

The first generation of competitive sEH inhibitors such as dicyclohexyl urea (DCU) and 1-adamantyl-3-cyclohexyl urea (ACU) were powerful transition-state mimics based on the urea moiety as a central pharmacophore, but they were very difficult to formulate (36–38). A previous study of TAC-induced cardiac hypertrophy involved 12-(3-adamantan-1-yl-



**Fig. 6.** Role of sEH knockout on Ang II-induced hypertrophy in cultured mouse NCMs. Murine NCMs were isolated from sEH<sup>-/-</sup> mice or their wild-type littermates (WT). Cell area and mRNA expression of  $\beta$ -MHC and ANF were determined on treatment with or without 100 nM Ang II (A2). Data are means  $\pm$  SD from 3 independent experiments (\*,  $P < 0.05$ ).

uredio) dodecanoic acid (AUDA) and 1-adamantan-1-yl-3-{5-[2[(2-ethoxy)ethoxy]pentyl]urea (AEPU) (36, 39); AUDA showed improved solubility but limited oral availability, and AEPU was even more soluble with excellent bioavailability, but both compounds were metabolized rapidly in rodents. TUPS was synthesized for this study on the basis of recently reported piperidine chemistry (40). Conformationally restricted TUPS retains the highly potent urea pharmacophore but has excellent oral availability and pharmacokinetic properties, so the compound is easier to use for testing the involvement of sEH in physiological processes (41).

Although cardiac hypertrophy can result from physiological exercise, mechanical overload, and/or neurohormonal factors, the mechanisms leading to hypertrophy during pathological and physiological states are distinct. Growth factors, such as insulin-like growth factor 1, are the most important cardiac factors involved in physiological hypertrophy, with phosphoinositide 3-kinase (PI3K) playing a critical role (42, 43). Mice with cardiac deletion of PI3K subunits exhibited attenuated Akt signaling in the heart, reduced heart size, and exercise-induced cardiac hypertrophy (42, 43). In pathophysiological hypertrophy, neurohormones, including NE and Ang II, play an important role to stimulate stress-mediated or -reactive cardiac hypertrophy and contribute to the progression to heart failure. However, the molecular events that signal hypertrophy are very complex and the signaling pathways for each neurohormone vs. renin-angiotensin in the cardiac hypertrophy response are different. Interestingly, sEH expression was upregulated only in the Ang II-induced, but not in the NE-induced pathologic state in this study. We previously reported that the protein level of sEH did not significantly change in TAC-induced hypertrophy in mice, but sEH inhibitor could prevent cardiac enlargement. The mechanisms leading to TAC-induced hypertrophy are more complicated, in which multiple pathways, including Ang II- and NE-mediated pathways, may be involved. It seems AT1-mediated Ang II signaling is not crucial since TAC could still induce cardiac hypertrophy in AT1 receptor knockout mice (44). NF- $\kappa$ B activation was inhibited by sEH in the TAC model (10), whereas the transcriptional upregulation of sEH by Ang II was exclusively via AP-1, which contributes to the pathological outcome of the Ang II-induced cardiac hypertrophy. The different response of cardiac Nox isoforms gp91<sup>phox</sup> and Nox4 to Ang II vs. TAC also suggested the difference between these two models (45). It is possible that TAC-induced hypertrophy is similar to induction by NE but not to that by Ang II (46, 47).

In summary, our findings define a mechanism of Ang II-induced hypertrophy that differs from physiological and other pathological hypertrophic models: sEH activity can be specifically upregulated by Ang II in cardiomyocytes *in vitro* and *in vivo*. sEH and its substrates/products, namely EETs/DHETs, potentially play a regulatory role in Ang II-induced maladaptive hypertrophy. Importantly, a potent sEH inhibitor could prevent the pathogenesis of cardiac hypertrophy. Thus, our findings may have clinical significance for the treatment of cardiac hypertrophy.

## Materials and Methods

**NCMs in Culture.** Primary cultures of rat NCMs were generated from 1- to 3-day-old Harlan Sprague-Dawley rat neonates and cultured as described previously (48). Murine NCMs were isolated from 3-day-old C57BL/6J or sEH<sup>-/-</sup> neonatal mice as previously described (49). The detailed methods of NCM isolation are shown in the online-only *SI*. Cardiomyocytes were maintained in serum-free DMEM for 24 h before adenoviral infection and/or treatments.

**Plasmid of Human sEH Promoter Transient Transfection.** The plasmids of the human sEH promoter sEH-1091-Luc (11) and a mutation construct of the sEH promoter, sEH-1091M-luc with the AP-1 site at -446 mutated, were used for transient transfection. Plasmid DNA was transfected into rat NCMs by use of the Superfect method (QIAGEN). CMV- $\beta$ -gal was cotransfected as a transfection control. After treatment, NCMs were lysed and the cell lysates were collected for luciferase activity assays.

**Adenovirus Construction and Infection.** Ad-sEH, a recombinant adenovirus expressing human Ephx2, was generated by subcloning the cDNA encoding Ephx2 (NM\_001979) into an adenoviral vector (Adeno-X Expression System2, Clontech). Confluent cardiomyocytes were infected with recombinant adenoviruses at the indicated multiplicity of infection (47) and incubated for 24 h before experimentation. Ad-GFP was used as a standard control.

**Western Blot Analysis, Quantitative Real-Time RT-PCR, and Immunohistochemistry.** See method in online-only *SI*.

**Animal Experiments.** All animal experimental protocols were approved by the Peking University Institutional Animal Care and Use Committee. Male SHRs and Wistar and Sprague-Dawley rats (all were within 180–280 g) (Peking University Health Science Center Animal Department) were kept in a 12-h light/12-h dark cycle at a controlled room temperature and had free access to standard chow and tap water. To induce hypertension, different models were used as described previously (48). For the induction of Ang II-infused hypertension with sEH inhibitor TUPS, Sprague-Dawley rats were divided into 4 experimental groups: 1 group received sham surgery, a second group Ang II infusion (450 ng/kg/min for 14 days), the third group Ang II and TUPS (oral gavage, 0.65 mg/kg/day, 7 days before surgery), and the fourth group only TUPS. TUPS (0.65 mg) was put in 1 ml PEG400 and vortexed until it completely dissolved. Then deionized water (3 ml) was added to the solution. The resulting solution (1 ml) was given to a 250-g rat. Ang II was infused at a continuous rate via an osmotic minipump (Alzet 1002). Then the rats were euthanized, and hearts were removed, blotted, and weighed to determine the ratio of heart weight to body weight. Systolic blood pressure (SBP) was determined every other day, beginning 1 day before the implantation of the minipumps for the duration of the study, by a computerized tail-cuff system (BP-2000, Visitech Systems).

**Analysis of Cardiac Function by Echocardiography.** See method in online-only *SI*.

**sEH Activity Assays.** The cells were scraped in 100  $\mu$ l extract buffer (0.1 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, with 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.1% Tween-20). The cytosolic supernatants were centrifuged at 1000 rpm for 5 min. 14,15-EET was added to a final concentration of 50  $\mu$ M. After incubation at 30 °C for 30 min, the reactions were terminated by adding 400  $\mu$ l methanol. After centrifugation, the DHETs and EETs in extracts were separated by reverse-phase HPLC on an ACQUITY UPLC BEH C18 column, 2.1  $\times$  100 mm, 1.7  $\mu$ m (Waters). DHETs and EETs were quantified by use of a 4000 QTRAP tandem mass spectrometer (Applied Biosciences) with negative-mode electrospray ionization and multiple reaction monitoring.

**Statistical Analysis.** Results are expressed as mean  $\pm$  SD from at least 3 independent experiments. The significance of variability was determined by an unpaired 2-tailed Student's *t*-test or ANOVA. Each experiment included triplicate measurements for each condition tested, unless indicated otherwise. *P* < 0.05 was considered statistically significant.

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