Supporting Information

Sirish et al. 10.1073/pnas.1221972110

SI Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and was approved by the University of California, Davis Institutional Animal Care and Use Committee.

Soluble Epoxide Hydrolase Inhibitor. The soluble epoxide hydrolase inhibitor (sEHI), 1-trifluoromethoxyphenyl-3-(1-propionylpiperidine-4-yl)urea (TPPU) was used in the study (Fig. 1*A*). The synthesis, physical properties, and spectral characteristics of TPPU were performed as previously described (1, 2).

Myocardial Infarction Model in Mice. All animal care and procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee. The myocardial infarction (MI) model in mice was created using procedures as previously described (3). Briefly, 10-wk-old male C57BL/6J mice (Charles River) were anesthetized with i.p. ketamine 80 mg/kg and xylazine 6 mg/kg. Intubation was performed perorally and mechanical ventilation was initiated. An oblique 4-mm incision was made 4 mm away from the left sternal border in the third–fourth intercostal space. The chest retractor was inserted and the heart, which was partially covered by the lung, was then visualized. The pericardium was gently picked up and pulled apart. The left anterior decending (LAD) coronary artery was then visualized and ligated 1–2 mm below the tip of the left auricle in its normal position. Occlusion was confirmed by the change of color of the anterior wall of the left ventricle (LV) and maintained for 45 min after which the occlusion was released. The sham-operated mice underwent the same procedure without tying the suture but moving it behind the LAD artery.

Echocardiograms were performed 1 wk after surgery after which mice were randomized to receive TPPU (15 mg/L) (2) in the drinking water or water alone for a period of 3 wk (Fig. 1*B*). Four groups of animals including sham, sham treated with TPPU, MI, and MI treated with TPPU were followed for a period of 3 wk at which time repeat echocardiograms were performed. The investigators were blinded to the treatment groups. A total of 104 male C57BL/6J mice were used. Eight animals died in the perioperative period. Seven animals were excluded from the study because of failure of the coronary artery occlusion, leaving a total of 89 mice in the study.

Thoracic Aortic Constriction Model in Mice. Thoracic aortic constrictions (TACs) were preformed in 8-wk-old male C57BL/6J (Charles River) mice as previously described (1). Briefly, animals were anesthetized with i.p. ketamine 50 mg/kg and xylazine 2.5 mg/kg. Intubation was performed perorally and mechanical ventilation was initiated. Aortic constriction was created via a left thoracotomy by placing a ligature securely around the ascending aorta and a 26-gauge needle and then removing the needle. The chest was closed with 3-0 dexon rib sutures, 5-0 dexon II muscle sutures, and buried skin sutures. Negative plural pressure was reestablished via a temporary chest tube until spontaneous breathing occurs. Sham-operated animals underwent the same procedure without tying the suture. Echocardiograms were performed 1 wk after surgery after which mice were randomized to receive TPPU (15 mg/L) (2) in the drinking water or water alone for a period of 3 wk. Four groups of animals including sham, sham treated with TPPU, MI, and MI treated with TPPU were followed for a period of 3 wk at which time repeat echocardiograms were performed.

Analysis of Cardiac Function by Echocardiography. Echocardiograms to assess systolic function were performed by using motion-mode (M-mode) and 2D measurements as described previously (4). The measurements represented the average of six selected cardiac cycles from at least two separate scans performed in random-blind fashion with papillary muscles used as a point of reference for consistency in the level of scan. Fractional shortening (FS), a surrogate of systolic function, was calculated from left ventricle dimensions as follows: $FS = ((EDD\text{-}ESD)/EDD) \times 100\%$, where EDD and ESD represent end-diastolic and end-systolic dimensions, respectively.

Histological Analyses. Hearts were excised and retrogradely perfused with phosphate-buffered solution to wash out blood and fixed in 10% (vol/vol) formalin overnight. Hearts were then embedded in paraffin, serial cardiac sections of 100 μm in thickness were taken along the longitudinal axis, and stained with Picrosirius Red to assess for collagen content. The percentage of infarcted area represents the ratio of connective tissues to total left ventricle (LV) area and was calculated by computerized planimetry (National Institutes of Health Image J software) (5). An observer blinded to the treatment groups performed measurements.

Immunofluorescence Confocal Laser Scanning Microscopy. Additional cardiac sections were stained with wheat germ agglutinin. The cardiac sections were deparaffinized with xylene before rehydrating with serial dilution of ethanol. The sections were blocked with donkey serum and stained with wheat germ agglutinin (10 $\mu g \cdot mL^{-1}$; Molecular Probes). Secondary antibodies conjugated to Alexa Fluor 488 were used. Cardiac sections from corresponding area from the four groups were scanned. Identical settings were used for all of the specimens.

FAC sorted $Lin^{-}/Thy1.2^{+}$ cells were plated on tissue culture dishes, fixed with 1% paraformaldehyde (PFA), and stained with anticollagen1a and antiprocollagen3a antibodies (Santa Cruz Biotechnology). Immunofluorescence-labeled and differential interference contrast (DIC) images were obtained using a Zeiss LSM700 confocal laser-scanning microscope.

Metabolomic Profiling of Oxylipins. Plasma samples stored at −80 °C were thawed at room temperature. Aliquots of plasma (200 μL) were spiked with a set of odd chain length analogs and deuterated isomers of several target analytes including hydroxyeicosatetraenoic acids (HETEs), prostaglandins, thromboxanes, epoxyoctadecenoic acids and epoxyeicosatrienoic acids (EpOMEs and EETs), and dihydroxyoctadecenoic acids and dihydroxyeicosatrienoic acids (DHOMEs and DHETs) contained in 10 μL of methanol, and then were extracted by solid phase extraction using Oasis HLB cartridges (Waters). The HLB columns (1 cc, 60 mg) were washed with 2 mL methanol and preconditioned with 2 mL water/methanol/acetic acid [95/5/0.1 (vol/vol)]. Samples were then mixed with 200 μL of the preconditioning solution and loaded onto the column. The loaded column was then washed with 2 mL of the preconditioning solution and then dried for 5 min in vacuo. Target analytes were then eluted with 2 mL of ethyl acetate. The collected eluents were evaporated to dryness using a centrifugal vacuum concentrator and redissolved in 40 μL of methanol. The spiked samples were vortexed for 1 min, centrifuged at 10,956 × *g* for 5 min, and then transferred to analytical vials containing 150 μL inserts for analysis.

The oxylipin profiling was performed using a modification of a previously published method (6). The separation of plasma oxylipins was conducted in a Shimadzu LC-10AD_{VP} instrument (Shimadzu) equipped with a 2.1 mm × 150 mm Pursuit XRs-C18 5-μm

column (Varian) held at 40 °C. A gradient of water containing 0.1% acetic acid [(vol/vol), solvent A] and acetonitrile/methanol/acetic acid [800/150/1 (vol/vol); solvent B] was used to elute the column with the flow rate of 0.4 mL·min^{-1} (Table S1). The injection volume was 10 μL and the samples were kept at 10 °C in the auto sampler. Analytes were detected on a 4000 QTRAP (Applied Biosystems) hybrid, triple-quadrupole, and linear ion trap mass spectrometer equipped with a Turbo V ion source and operated in negative multiple reaction monitoring mode. The source was operated in negative electrospray mode and the QTRAP was set as follows: CUR= 20 psi, GS1 = 50 psi, GS2 = 30 psi, IS = −4,500 V, CAD = high, TEM = 400 °C, ihe = on, DP = −60 V, where CUR, GS1, GS2, IS, CAD, TEM, ihe, and DP refer to Curtain Gas, Ion Source Gas 1, Ion Source Gas 2, IonSpray Voltage, CAD Gas, Temperature, ihe: 1 = ON and 0 = OFF, and Declustering Potential, respectively. The collision energies used for CAD ranged from −18 to −38 eV.

Measurement of Plasma Cytokine Levels. Plasma samples were collected 3 wk after sham or MI operation and stored at −70 °C until assayed. Plasma cytokine levels were analyzed using a Cytometric Bead Array kit (CBA mouse inflammation kit; BD Biosciences). Briefly, thawed plasma samples were mixed for 2 h at room temperature with florescence-labeled capture beads with the PE detection reagents to measure the concentrations of interleukin-6 (IL-6), interleukine-1β (IL-1β), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and interleukin-12p70 (IL-12 p70). Samples were then washed with washing buffer and analyzed on a FACScan flow cytometer (BD Immunocytometry Systems). Data were analyzed using BD Cytometric Bead Array Analysis software (BD Immunocytometry Systems).

Flow Cytometric Analysis of Nonmuscle Cells. Single-cell suspension was obtained from 8- to 12-wk-old male C57BL/6 mice as previously described (7). The procedure was performed according to the approved University of California Davis Animal Care and Use protocol. Briefly, mice were injected with 0.1 mL heparin (1,000 units·mL^{-1}) 10 min before heart excision and then anesthetized with pentobarbital intraperitoneally (80 mg·kg^{-1}). Hearts were removed and placed in Tyrode's solution (mmol·L^{-1}: NaCl 140, KCl 5.4, $MgCl_2$ 1.2, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes) 5 and glucose 5, pH 7.4). All chemicals were obtained from Sigma Chemicals unless stated otherwise. The aorta was cannulated under a dissecting microscope and mounted on the Langendorff apparatus. The coronary arteries were retrogradely perfused with Tyrode's solution gassed with O_2 at 37 °C for 3 min at a flow rate of ∼3 mL·min^{-1}. The solution was switched to Tyrode's solution containing collagenase type 2 (1 mg·mL^{-1}, 330 units·mg^{-1}; Worthington Biochemical). After ∼12 min of enzyme perfusion, hearts were removed from the perfusion apparatus and gently teased in high-K^+ solution (mmol·L^{-1}: potassium glutamate 120, KCl 20, $MgCl_2$ 1, EGTA 0.3, glucose 10 and Hepes 10, pH 7.4 with KOH). The cells were filtered through a 200-μm cell strainer, resuspended in Ca^{2+} and Mg^{2+} free PBS, treated with phytoerythrin-conjugated anti-Thy1.2 (BD Bioscience), anti–FSP-1 (Millipore), lineage antibody mixture (CD3e, CD11b, CD45R, Ly-6C (lymphocyte antigen), Ly-6G, and Ly76, 1:100 dilution; BD Bioscience), anti-CD34, anti-CD45 (BD Bioscience), anti-troponin T antibody (Thermo Scientific), anti-CD31 (BD Bioscience), Alexa Fluor 488 conjugated anti-phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) (Cell Signaling), anti-extracellular signal-regulated kinase 1/2 (ERK1/2) (Cell Signaling), anti–beta-myosin heavy chain (-β-MyHC) (clone NOQ7.5.4D; Sigma), and proliferation-specific Ki67 antibody (15 μg·mL^{-1}; BD Bioscience) in PBS with 5% donkey serum and 20 μg·mL^{-1} DNase-free RNase (Sigma) overnight at 4 °C (8). Cells were also stained with 40 μg·mL^{-1} 7-amino-actinomycin D (7AAD; BD Bioscience) to measure the DNA content. Data were collected using a standard FACScan cytometer (BD Biosciences) upgraded to a dual laser system with the addition of a blue laser (15 mW at 488 nm) and a red laser (25 mW at 637 nm; Cytek Development) or Becton Dickinson LSR-II Flow cytometer. Data were acquired using CellQuest and DIVA 6.2 software (BD Bioscience). Cells stained with isotype-matched IgG antibodies were used as controls to determine the positive cell population. Data were analyzed using FlowJo software (version 9.4 Treestar).

In Vitro Cardiac Fibroblast Culture. After obtaining the single cell suspension as described above, the cells were plated on a 100-mm culture dish, incubated at 37 °C for 45 min. The culture dish was washed two times with medium and cultured until the cells reached confluency. Cells were treated with angiotensin II (AngII) (1 μM) alone or with TPPU (1 μM) for 30 min before flow cytometric analysis of Thy^{pos} ($Thy1.2+/Lin^-/CD31^-/CD34^-/CD45^-$) cells.

Western Blot Analysis. Immunoblots were performed as previously described (9). The following primary antibodies were used: (*i*) Rabbit monoclonal antiphospho-p44/42 mitogen-activated protein kinase (MAPK) antibody (1:2,000 dilution; Cell Signaling), (*ii*) rabbit monoclonal anti-p44/42 MAPK antibody (1:1,000 dilution; Cell Signaling), (*iii*) polyclonal antiatrial natriuretic polypeptide (1:2,000; Millipore), and (*iv*) monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:10,000 dilution; Fitzgerald Industries) was used as an internal loading control.

Myocyte Volume Measurements by Coulter Multisizer. The isolated cells were fixed with 0.4% (wt/vol) PFA for 10 min and were analyzed using Coulter Multisizer 4 as previously described (10). At least 10,000 myocytes were assayed and the mean volume distribution was calculated from the distribution curves generated by the Coulter Multisizer 4 software.

Semiquantitative RT-PCR Analysis. Total RNA was isolated from FAC-sorted Lin^- (using lineage antibodies mixture directed against CD3e, CD11b, Cd45R, Ly-6C, Ly-6G, and Ly76)/$Thy1.2^+$ cells using RNA isolation kit (Qiagen) according to the manufacturer's protocol. A total of 1 mg of total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) with Oligo dT primers (Invitrogen) according to the manufacturer's protocol. cDNA was diluted with water (1:1), used for PCR amplification for 35 cycles with gene-specific primers and separated on a 1.5% agarose gel. Photography of the gel and quantification of the intensity of the ethidium bromide-stained bands were performed by using the Fluorchem 8000 imaging system (Alpha Innotech). GAPDH was used as an internal standard to compare the expression levels of the tested genes using the following primers.

Gene	Primers
Collagen 1a	F: CCCACCCCAGCCGCAAAGAG
	R: GGGGCCAGGCACGGAAACTC
Collagen 3a	F: TGCAGGGTCCCCTGGCTCAA
	R: GGAACCAGCTTCGCCCCGTT
Nkx2.5 transcription factor	F: GAGCCTACGGTGACCCTGACCCAG
	R: TGACCTGCGTGGACGTGAGCTTCA
Von Willebrand factor	F: GCCTGGGGCCTCCAAAGCAG
	R: CCCGTGCACACACAAGGGCA
Platelet endothelial cell adhesion molecule	F: AGCCTCACCAAGCTCTGGGAAC
	R: TGGGCCTTCGGCATGGAACG
GAPDH	F: ACCACAGTCCATGCCATCAC
	R: TCCACCACCCTGTTGCTGTA

Statistical Analysis. Statistical comparisons were analyzed by Student *t* tests or one-way ANOVA followed by Bonferroni tests for post hoc comparison. Statistical significance was considered to be achieved when $P < 0.05$.

1. Xu D, et al. (2006) Prevention and reversal of cardiac hypertrophy by soluble epoxide hydrolase inhibitors. *Proc Natl Acad Sci USA* 103(49):18733–18738.
2. Hwang SH, Tsai HJ, Liu JY, Morisseau C, Hammock BD (2007) Orally bioavailable potent soluble epoxide hydrolase inhibitors. *J Med Chem* 50(16):3825–3840.
3. Tarnavski O, et al. (2004) Mouse cardiac surgery: Comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiol Genomics* 16(3):349–360.
4. Li N, et al. (2009) Beneficial effects of soluble epoxide hydrolase inhibitors in myocardial infarction model: Insight gained using metabolomic approaches. *J Mol Cell Cardiol* 47(6):835–845.
5. Dawn B, et al. (2006) Postinfarct cytokine therapy regenerates cardiac tissue and improves left ventricular function. *Circ Res* 98(8):1098–1105.
6. Morisseau C, et al. (2002) Structural refinement of inhibitors of urea-based soluble epoxide hydrolases. *Biochem Pharmacol* 63(9):1599–1608.
7. Sirish P, et al. (2012) MicroRNA profiling predicts a variance in the proliferative potential of cardiac progenitor cells derived from neonatal and adult murine hearts. *J Mol Cell Cardiol* 52(1):264–272.
8. López JE, et al. (2011) β-myosin heavy chain is induced by pressure overload in a minor subpopulation of smaller mouse cardiac myocytes. *Circ Res* 109(6):629–638.
9. Xu Y, et al. (2005) The effects of intracellular Ca^{2+} on cardiac K^{+} channel expression and activity: novel insights from genetically altered mice. *J Physiol* 562(Pt 3):745–758.

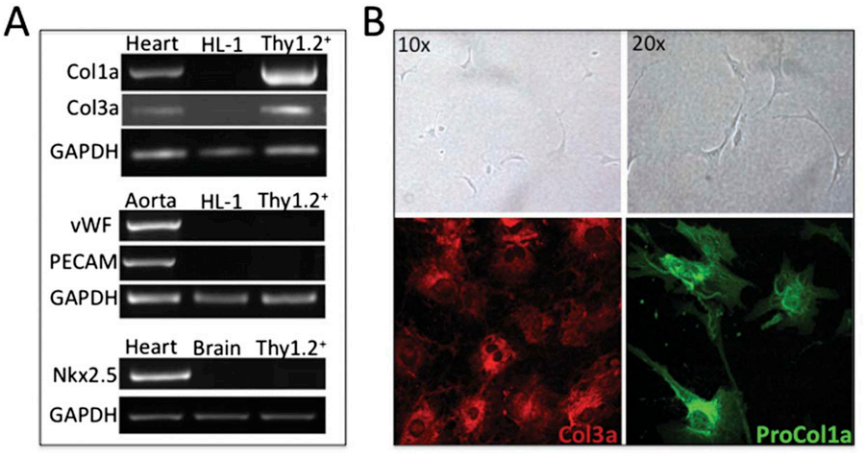

Fig. S1. Characterization of fluorescence-activated cell sorting of Thy1.2^{+} cells. (*A*) Semiquantitation RT-PCR analysis. Lane 1, positive control; lane 2, negative control (HL-1 cells and brain); lane 3, Thy1.2^{+} cells. Col1a, collagen 1a; Col3a, collagen 3a; and vWF, von Willebrand factor. (*B*) Photomicrographs of FAC-sorted Thy1.2^{+} cells at 10× and 20× magnification. Below, immunofluorescence confocal images of Thy1.2^{+} cells showing collagen 3a (red) and procollagen 1a (green) staining.

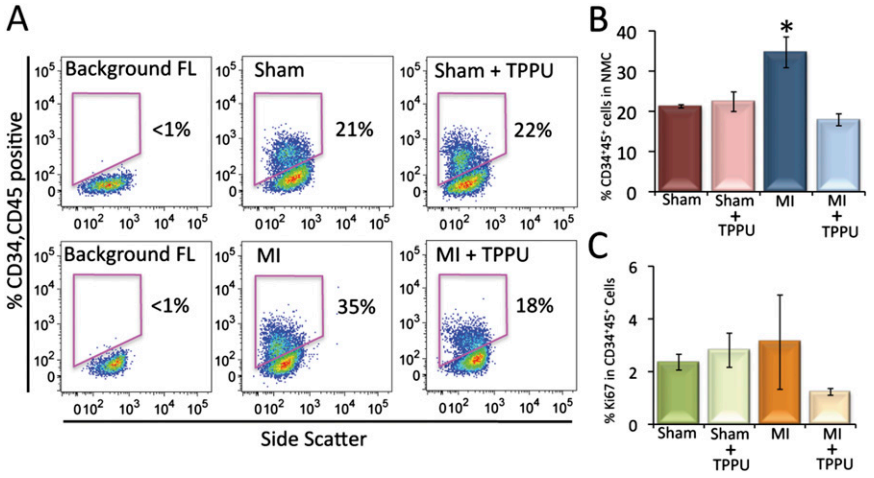

Fig. S2. (*A*) Flow cytometric analysis of CD34^{+}CD45^{+} single and double positive cells from the four groups of animals including sham ± TPPU, and MI ± TPPU. (*B*) Summary data from *A* ($n = 3$ per group). (*C*) Summary data showing the proliferative CD34^{+}CD45^{+} cells using Ki67 proliferative marker. Representative results are shown. Data shown represent mean ± SE and $^{*}P < 0.05$.

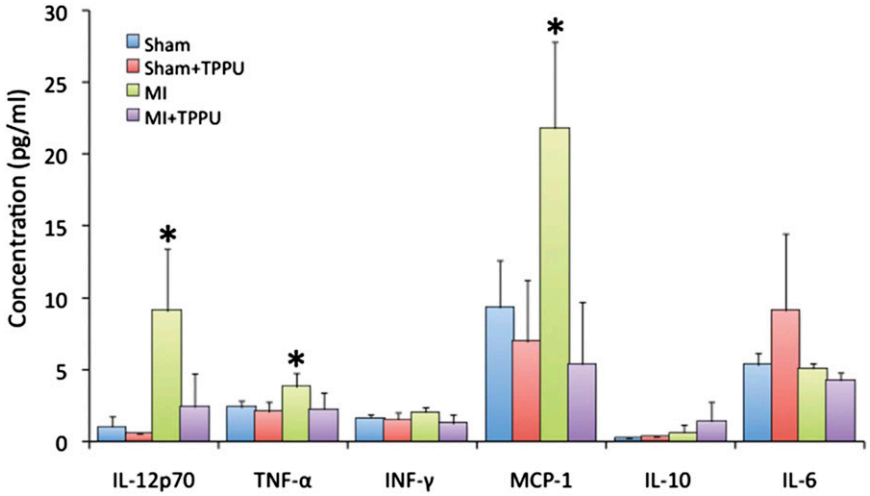

Fig. S3. Serum concentration (picograms per milliliter) of cytokines from the four groups of animals including sham ± TPPU, and MI ± TPPU. Data shown represent mean ± SE and $^{*}P < 0.05$.

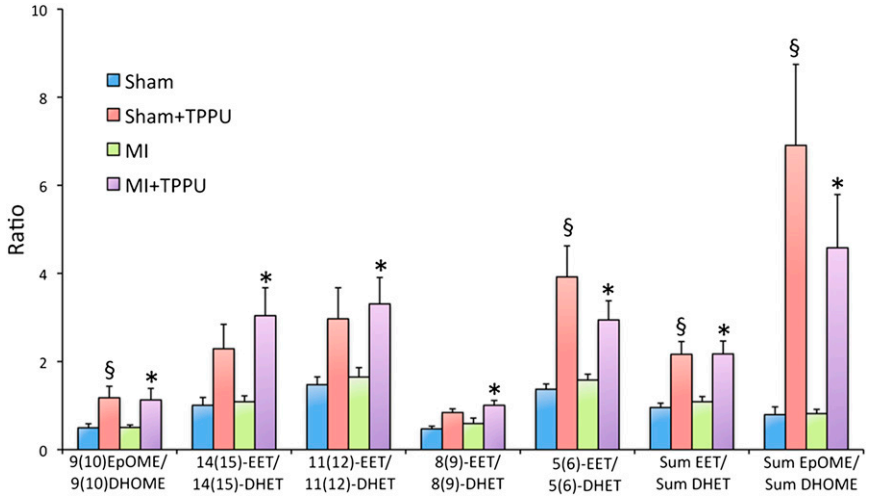

Fig. S4. Oxylipin profiling from the four groups of animals including sham ± TPPU, and MI ± TPPU at 3 wk of follow-up. Data shown represent mean ± SE and $^{*}P < 0.05$ comparing MI + TPPU from sham ± TPPU or MI groups, $^{\S}P < 0.05$ comparing sham + TPPU from sham or MI ± TPPU groups.

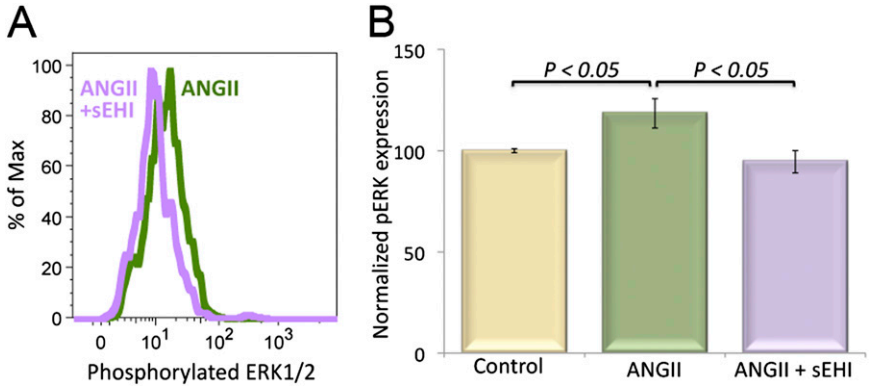

Fig. S5. (*A*) Flow cytometric analysis showing pERK1/2 signal in cultured cardiac mouse Thy^{pos} fibroblasts treated with angiotensin II (ANGII, 1 μM, green) alone and with TPPU (1 μM, purple) for 30 min. (*B*) Summary data from *A* showing a significant increase in pERK1/2 levels in fibroblasts treated with AngII (green) compared with control (yellow) and a significant decrease in the pERK1/2 levels in the TPPU-treated cells (purple).

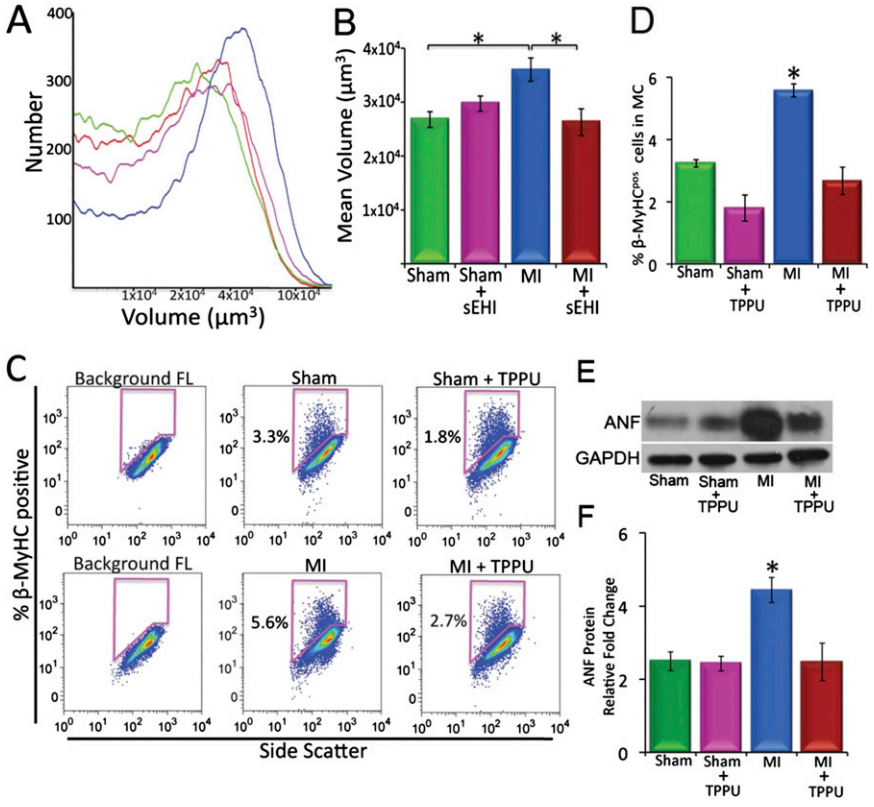

Fig. S6. TPPU in the MI model prevents cardiac myocyte hypertrophy as assessed by cell volume and hypertrophic markers. (*A*) Graph showing the myocyte volume measurements by the Coulter Multisizer assay. Green curve (sham operated), pink curve (TPPU treated, sham operated), blue curve (MI), and red curve (TPPU-treated MI). (*B*) Summary data from *A* ($n = 3$ per group). (*C*) Flow cytometric analysis of β-MyHC expression in myocytes in four groups. (*D*) Summary data from *C*. (*E*) Representative lanes of Western blot assay and (*F*) summary data for atrial natriuretic factor (ANF) and GAPDH (loading control) from whole cell lysates from the four groups ($n = 3$ per group).

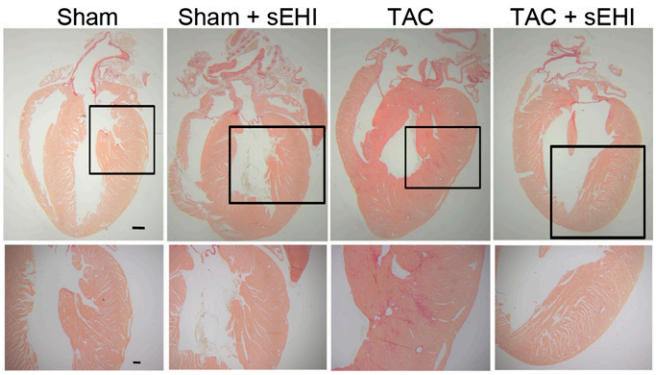

Fig. S7. Examples of cardiac sections stained with Sirius Red from sham-operated, sEHI-treated–sham-operated, MI and sEHI-treated MI mice. The mice were killed after 3 wk of follow-up. (Scale bars, 200 μm.)

Table S1. Summary of echocardiographic data at 3 wk after MI

Treatment	*N*	EDD, cm	ESD, cm	LVPW (D), cm	LVPW (S), cm	FS, %
Sham	15	0.33 ± 0.01	0.15 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	54.9 ± 1.8
Sham + TPPU	10	0.34 ± 0.01	0.16 ± 0.01	0.11 ± 0.005	0.15 ± 0.01	53.6 ± 2.6
MI alone	19	0.36 ± 0.01	0.20 ± 0.01*	0.09 ± 0.005	0.12 ± 0.005*	44.0 ± 1.3*
MI + TPPU	20	0.36 ± 0.01	0.18 ± 0.01†	0.09 ± 0.005	0.13 ± 0.005†	50.0 ± 1.4†

EDD, end diastolic dimension; ESD, end systolic dimension; LVPW (D), left ventricular posterior wall thickness in diastole; LVPW (S), left ventricular posterior wall thickness in systole. Data are mean ± SEM (one-way ANOVA followed by Bonferroni tests for post hoc comparison, *$P < 0.05$ comparing MI alone with sham, $^{\dagger}P < 0.05$ comparing treated vs. untreated MI animals).