Supplementary data

Supplementary methods

Cell culture:

(1) Neonatal rat cardiac fibroblasts cultures:

Neonatal rat cardiac fibroblasts were isolated as described in manuscript, detailed treatments of additional experiments were presented as below：

Treatment 1: Cardiac fibroblasts (3×10^5 cells per well) were serum-starved overnight, and then incubated with Ang-II (1μM) in the presence or absence of different EET regioisomer (1μM, Cayman Chemical, Ann Arbor, MI) for 24 hours.

Treatment 2: Cardiac fibroblasts (3×10^5 cells per well) were serum-starved overnight, and then incubated with Ang-II (1μM) in the presence or absence of different does pf 11,12-EET (0.5nM to 1μM, Cayman Chemical, Ann Arbor, MI) for 24 hours.

Treatment 3: Cardiac fibroblasts (3×10^5 cells per well) were serum-starved overnight, and then incubated with different does of Ang-II (0nM to 2μM) for 24 hours.

Treatment 4: Cardiac fibroblasts (3×10^5 cells per well) were serum-starved overnight, and then incubated with Ang-II (1μM) for various incubation time.

Treatment 5: Cardiac fibroblasts (3×10^5 cells per well) were serum-starved overnight, and then incubated with Ang-II (1μM) in the presence or absence of 11,12-EET for various incubation time.

Treatment 6：Cardiac fibroblast (3×10^5 cells per well) were serum-starved overnight, and then incubated with Ang-II (1μM) in the presence or absence of different does of

11,12-DHET (0.3-3μM, Cayman Chemical, Ann Arbor, MI) for 24 hours.

Treatment 7：Cardiac fibroblasts (3×10^5 cells per well) were serum-starved overnight, and then incubated with Ang-II (1μM) in the presence or absence of 17,18-EEQ (1μM, Cayman Chemical, Ann Arbor, MI) for 24 hours.

(2) Neonatal mouse cardiomyocytes isolation and cultures:

Neonatal CYP2J2 transgenic mice and C57BL/6 mice were anesthetized with 4–5% isoflurane inhalation anesthesia. Adequate anesthesia was determined by the absence of reflexes prior to rapid heart excision. Neonatal cardiomyocytes were isolated as described previously[1]. Briefly, hearts were excised from 1–2-day-old WT or CYP2J2-Tr mice, minced, and digested in 0.06% (w/v) trypsin (Invitrogen) and 0.025% (w/v) collagenase type II (Invitrogen) in calcium-free Hanks' buffer (with HEPES) by serial digestion. Cells were centrifuged and resuspended in Dulbecco's modified Eagle's medium: F12 medium (Invitrogen) with 10% (v/v) fetal calf serum and 1% penicillin/streptomycin (Invitrogen). Cardiac fibroblasts were separated from cardiomyocytes by differential adhesion. Cells were plated on cell culture flask for 90 min (37 °C, 5%CO2), during the time, fibroblasts adhere to the flask but not the cardiomyocytes. Cardiomyocytes were collected and equal cell suspension was added in six well plate (3×10^5 cells per well) in the presence of Brdu (Sigma, Saint Louis, MO) to reduce the number of nonmyocytic cells. Cardiomyocytes were cultured for 3-4 days before use.

Cardiomyocytes from WT mice or transgenic mice were incubated with Saline or Ang-II (1μmol/l) for 6 hours after overnight serum-starvation and then cultured medium were collected for eicosanoids detection.

Eicosanoids detection:

Metabolites of arachidonic acid and Linoleic acid in cardiac tissue, plasma and cultured medium were measured by LC/MS/MS as described previously (1, 2). Detailed information including sample preparation, LC/MS/MS condition and precision and accuracy of this method was also described previously (1, 2). Detailed protocol for Sample preparation and LC/MS/MS condition were listed below：

(1) Preparation of plasma for eicosanoids measurements:

Prepare a 20% KOH solution by mixing 1 volume of 2M KOH and 4 volume of methanol (final concentration KOH = 0.4 N). To cleave the esterified eicosanoids, 2 mL of 20% KOH was added and mixed very well. The mixture was incubated at 50°C for one hour. Add 1.5 fold of H_2O to the solution and adjust pH with 20% formic acid to 5. Add ethyl acetate (1:1 ratio of aqueous solution and ethyl acetate), vortex thoroughly, and centrifuge at 4000 rpm for ten minutes at 4ºC. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Pool all the organic phase (ethyl acetate) together and add internal standard mixture, then evaporate to dryness. The residue was dissolved in 100µl 30% acetonitrile. After vigorous mixing, samples were filtered into vials of an auto-sampler through a 0.22-µm membrane and were then applied to LC-MS/MS as described below.

(2) Preparation of heart tissue for eicosanoids measurements:

Up to 50 mg heart tissue was added to a 2 ml micro-centrifuge tube with 100ul methanol and 5ng internal standard. The heart tissue in tube surround with crushed ice was homogenized using a micro-ultrasonic cell disrupter at 30 amplitudes (2 mm probe, highest power 100 amplitudes, Kontes), Dilute the homogenate to 2ml with methanol, vortex

thoroughly, and centrifuge at 4000 rpm for ten minutes at 4°C, The supernatant was transferred to a fresh 15ml tube. To cleave the esterified eicosanoids, 500ul 2MKOH was added and mixed very well. The mixture was incubated at 50°C for one hour followed by centrifuging at 12,000 rpm for 10 min at 4◦C. The supernatant was transferred to a fresh 15ml tube. Add 1.5 fold of H_2O to the solution and adjust pH with 20% formic acid to 5. Add ethyl acetate (1:1 ratio of aqueous solution and ethyl acetate), vortex thoroughly, and centrifuge at 4000 rpm for ten minutes at 4ºC. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. All the organic phase (ethyl acetate) was collected together and was evaporated to dryness. The residue was dissolved in 100μl 30% acetonitrile. After vigorous mixing, samples were filtered into vials of an auto-sampler through a 0.22μm membrane and were then applied to LC-MS/MS as described below.

(3) Preparation of cultured medium for lipidomic measurements:

Briefly, cultured mediums of cardiomyocytes from αMHC-CYP2J2 Tr mice or C57BL/6 mice were obtained for 6 hours incubation with Ang II or saline. Cultured medium was extracted by solid-phase extraction (SPE). Before extraction, Waters Oasis-HLB cartridges were washed with methanol (2 mL), ethyl acetate (2ml) and 95:5 v/v water/methanol (1.5ml). Samples were spiked with internal standard mixture. Cultured medium was loaded onto cartridges directly. Cartridges were washed with 1.5 mL of 5% methanol. The aqueous plug was pulled from the SPE cartridges by high vacuum, and SPE cartridges were further dried by low vacuum for about 20 min. SPE cartridges were eluted with 0.6mL methanol twice followed by 0.75ml of ethyl acetate into 2mL tubes. The eluent was then evaporated to dryness. The residue was dissolved in 100μl 30% acetonitrile. After vigorous mixing, samples

were filtered into vials of an auto-sampler through a 0.22 μm membrane and were then applied to LC-MS/MS as described below.

(4) HPLC and MS Conditions:

Targeted eicosanoids involved use of a 5500 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (AB Sciex, Foster City, CA) equipped with a turbo ion spray electrospray ionization source. The mass spectrometer was operated with Analyst 1.5.1 software. Analytes were detected by MRM scans in negative mode. The dwell time used for all MRM experiments was 25 ms, the ion source parameters were CUR = 40 psi, GS1 = 30 psi, GS2 =30 psi, IS = −4500 V, CAD = MEDIUM, and TEMP = 500 °C.

Protocol for $G\alpha_{12/13}$ activity assay and RhoA activity assay

1. GST-fusion protein preparation for pull down assay:

(1) GST-TPR were prepared for $G\alpha_{12/13}$ activity assay: cDNA encoding PP5-TPR with BamHI and EcoRI sites were generated by PCR from mouse cDNA using primers 5'-CGGGATCCGAGTGTGCTGAGACCCCCC-3' and 5'-CGGAATTCATCTTGGCATCCTTGTCATTAG-3' and subcloned into BamHI /EcoRI sites of PGEX-6p-1 vector, respectively. Recombinant proteins were expressed as GST-TPR fusion proteins in BL21 (DE3) pLysS Chemically Competent Cells *(*Transgene biotech) and purified on glutathione-Sepharose beads according to the method of Matsuiet al **(3)**. Protein concentration was determined by comparing with bovine serum albumin standards after electrophoresis on a SDS polyacrylamide gel and staining with coomassie brilliant blue.

(2) GST-fused Rho-binding domain of Rhotekin was purchased from cytoskeleton. Inc for

RhoA activity assay:

2. Sample preparation and extraction for detecting activities of RhoA and $G\alpha_{12/13}$:

Grow cells in appropriate culture conditions. Cells were treated with various regents for 5mins. After treatment, place culture vessel on ice, aspirate media, wash with ice cold PBS. Aspirate off PBS, tilt plates on ice for an additional 1 min to remove all remnants of PBS. Residual PBS will adversely affect the assay. Lyse cells in an appropriate volume of ice-cold cell lysis Buffer: lysis buffer for RhoA detection should be supplemented with 1X Protease Inhibitor Cocktail: (10 mM MgCl2, 25 mM HEPES pH7.5, 10% Glycerol, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630). 100X Protease Inhibitor Cocktail solution: 62 µg/ml Leupeptin, 62 µg/ml Pepstatin A, 14 mg/ml Benzamidine and 12 mg/ml tosyl arginine methyl ester. Lysis buffer for $G\alpha_{12/13}$ activity assay : 20 mM Hepes, 0.1% Triton X-100, pH 8.0, 2 mM $MgCl_2$,1 mM EDTA, 10 g/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10g/mL leupeptin. Harvest cell lysates with a cell scraper. It is useful to incline the culture plate for this method because the lysis Buffer is spread thinly on the surface. Transfer lysates into the pre-labeled sample tubes on ice. Immediately clarify by centrifugation at 10,000 x g, 4°C for 1 min. At this point each lysate volume should not exceed 130% of the original cell lysis Buffer volume. Save at least 20 µl of lysate for protein quantitation and 20-50 µg of lysate for Western blot of total RhoA. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Then store cell lysates at -70°C for future use.

3. Pull down assay:

Add equivalent protein amounts of lysate (300µg total cell protein) to a pre-determined amount of beads. For $G\alpha_{12/13}$ activity assay: add to GST-TPR beads, for RhoA activity assay :

add to GST-RBD beads. Then incubate at 4°C on a rotator or rocker for 1-2 h. Pellet the beads by centrifugation at 3-5,000 x g at 4°C for 1 min. Very carefully remove 90% of the supernatant. Do not disturb the bead pellet. Wash the beads once with 500 µl each of Wash Buffer (25 mM Tris pH 7.5, 30 mM MgCl2, 40 mM NaCl). Pellet the beads by centrifugation at 3-5,000 x g at 4°C for 3 min. Very carefully remove the supernatant. Add 10-20 µl of 2x Laemmli sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% betamercaptoethanol) to each tube and thoroughly resuspend the beads. Boil the bead samples for 2 min. The samples are now ready to be analyzed by SDS-PAGE and Western blot analysis.

4. Pull down protein detection (Western Blot Protocol).

Run the test protein samples on a 10% SDS gel until the dye front reaches the bottom of the gel. Equilibrate the gel in Western blot buffer (See recipe below) for 15 min at room temperature prior to electro-blotting. Transfer the protein to a PVDF membrane. Wash the membrane once with TBST. Block the membrane surface with 5% nonfat-dry milk in TBST for 30 min at room temperature with constant agitation. Incubate the membrane with 1:1000 dilution of anti-$G\alpha_{12}$ antibody (Santa Cruz Biotechnology, Inc. sc-409), 1:1000 dilution of anti-$G\alpha_{13}$ antibody (Santa Cruz Biotechnology, Inc. Sc-410) and 1:1000 dilution of anti-RhoA antibody (Abcam, ab187027) for 2-3 h at room temperature or overnight at 4°C with constant agitation. Rinse the membrane in 50 ml TBST for 1 min. Incubate the membrane with an appropriate dilution of secondary antibody in TBST for 30 min-1 h at room temperature with constant agitation. Wash the membrane 5 times in TBST for 10 min each. Use an enhanced chemiluminescence detection method to detect the $G\alpha_{12}$, $G\alpha_{13}$ and RhoA signal.

NO detection:

Cardiac fibroblasts were treated with various regents for 5 mins. Cells were lysed and the samples were centrifuged at 100,000xg to remove particulates and membrane proteins. For cardiac tissue sample, homogenized the sample in PBS (PH 7.4), and centrifuged homogenates 10000g for 20 mins. Centrifuge at 100000g for 30 mins, ultrafilter homogenates through a 10 or 30KD molecular weight cut off filter. NO were detected as manufacture's instruction (Cayman Chemical, Ann Arbor, MI). Briefly, aliquot the standards for standard curve, then aliquot samples to the wells and adjust the volume to 80µl with assay buffer. Add 10µl of the enzyme co-factor mixture to each well. Add 10µl of nitrate reductase mixture to each well. Incubate the plate at room temperature for 30mins (for tissue samples, incubate 2h). Add 10µl DAN reagents to each well and incubate for 10mins. Add 20µl of NaOH to each well. Read the plate using the fluorometer using an excitation wave length of 360-365nm and emission wave length of 375 and 417nm. The NO concentrations were normalized to the protein content.

cGMP Measurements

(1) Sample preparation for cellular cGMP detection: Aspirate medium for plate, add 1ml of 0.1M HCL for every 35 cm^2 of surface area. Incubate at room temperature for 20 minutes. Scrape cells with a cell scraper. Dissociate the mixture and transfer to centrifuge tube. Centrifuge at 1000g for 10 minutes. Transfer the supernatant into a clean test tube.

(2) Sample preparation for tissue cGMP detection: weigh the frozen tissue and drop into 5-10 volume of 5% trichloroacetic acid in water. Homogenize the sample on ice using the

polytron-type homogenizer. Remove the precipitate by centrifugation at 1500g for 15 minutes and transfer the supernatant into a clean test tube. Extract TCA from the sample using water saturated ether, repeat extraction two more times. Remove residual ether from the aqueous layer by heating the sample to 70℃ for 5 minutes.

(3) cGMP were detected as manufacture's instruction (Cayman Chemical, Ann Arbor, MI). Briefly, Prepare for standard curve, then add ELISA buffer, cGMP standard, sample, cGMP AChE tracer and cGMP ELISA anti-serum to respective wells. Incubate the plate 18 hours at troom temperature and read the plate at wave length between 405 and 420 nm. Finally, calculate relevant cGMP levels.

Antibodies information:

CYP2J2 (Abcam, ab151996**,** 1:1000 for WB); COL1A (Santa Cruz, sc-8784-R, 1:1000 for WB, 1:200 for IHC-P); α-SMA (Abcam, ab7817, 1:300 for WB, 1:200 for IF, 1:200 for IHC-P); Vimentin (Abcam, ab8978, 1:1000 for WB, 1:500 for IF); β-actin (Abcam, ab8226**,** 1:1000 for WB); PCNA (proteintech, 10205-2-AP, 1:1000 for WB,1:200 for IF); MRTF-A (Santa Cruz, sc32909, 1:1000 for WB); LaminB1 (Santa Cruz, sc-377000, 1:1000 for WB); RhoA (Abcam, ab187027, 1:1000 for WB); G12 (Santa Cruz, sc-409, 1:1000 for WB); G13 (Santa Cruz, sc-410, 1:1000 for WB); P-MYPT1(Thr696) (Cell Signaling, #5163 1:1000 for WB); MYPT1 (Cell Signaling, #2634, 1:1000 for WB); p-PKCα (Santa Cruz, sc-377565, 1:1000 for WB); PKCα (Santa Cruz, sc-12356-R, 1:1000 for WB); CTGF (Santa Cruz, sc-365970, 1:1000 for WB); p-PLCβ3 (Abcam, ab73998 1:1000 for WB); PLCβ3 (Abcam, ab52199, 1:1000 for WB)

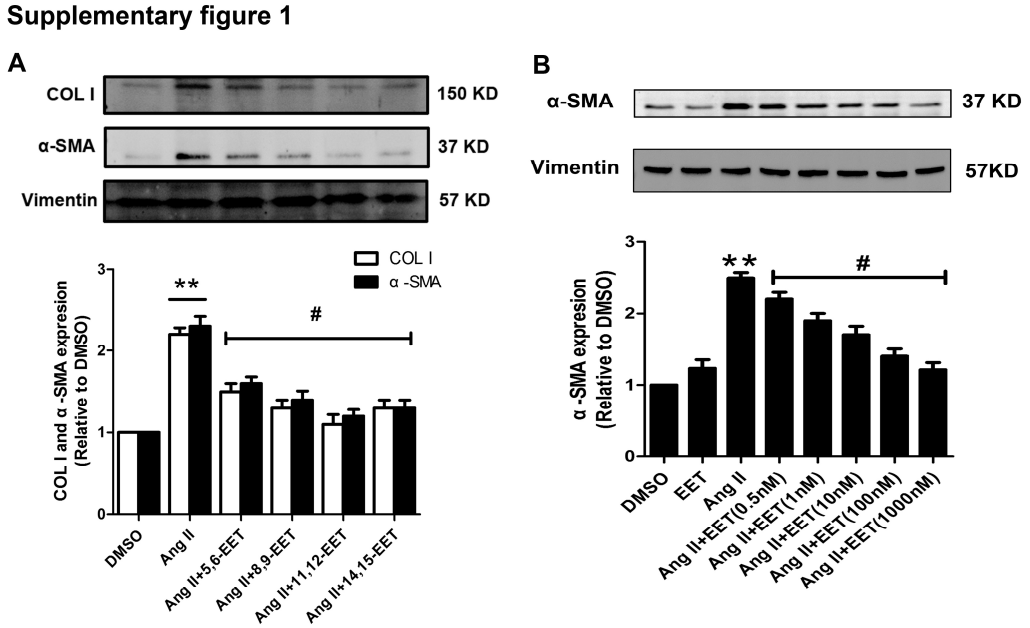
Supplementary References

1. Zhang, X., N. Yang, D. Ai, and Y. Zhu. 2015. Systematic metabolomic analysis of eicosanoids after omega-3 polyunsaturated fatty acid supplementation by a highly specific liquid chromatography-tandem mass spectrometry-based method. *J Proteome Res* **14**: 1843-1853.

2. Li, L., N. Li, W. Pang, X. Zhang, B. D. Hammock, D. Ai, and Y. Zhu. 2014. Opposite effects of gene deficiency and pharmacological inhibition of soluble epoxide hydrolase on cardiac fibrosis. *PLoS One* **9**: e94092.

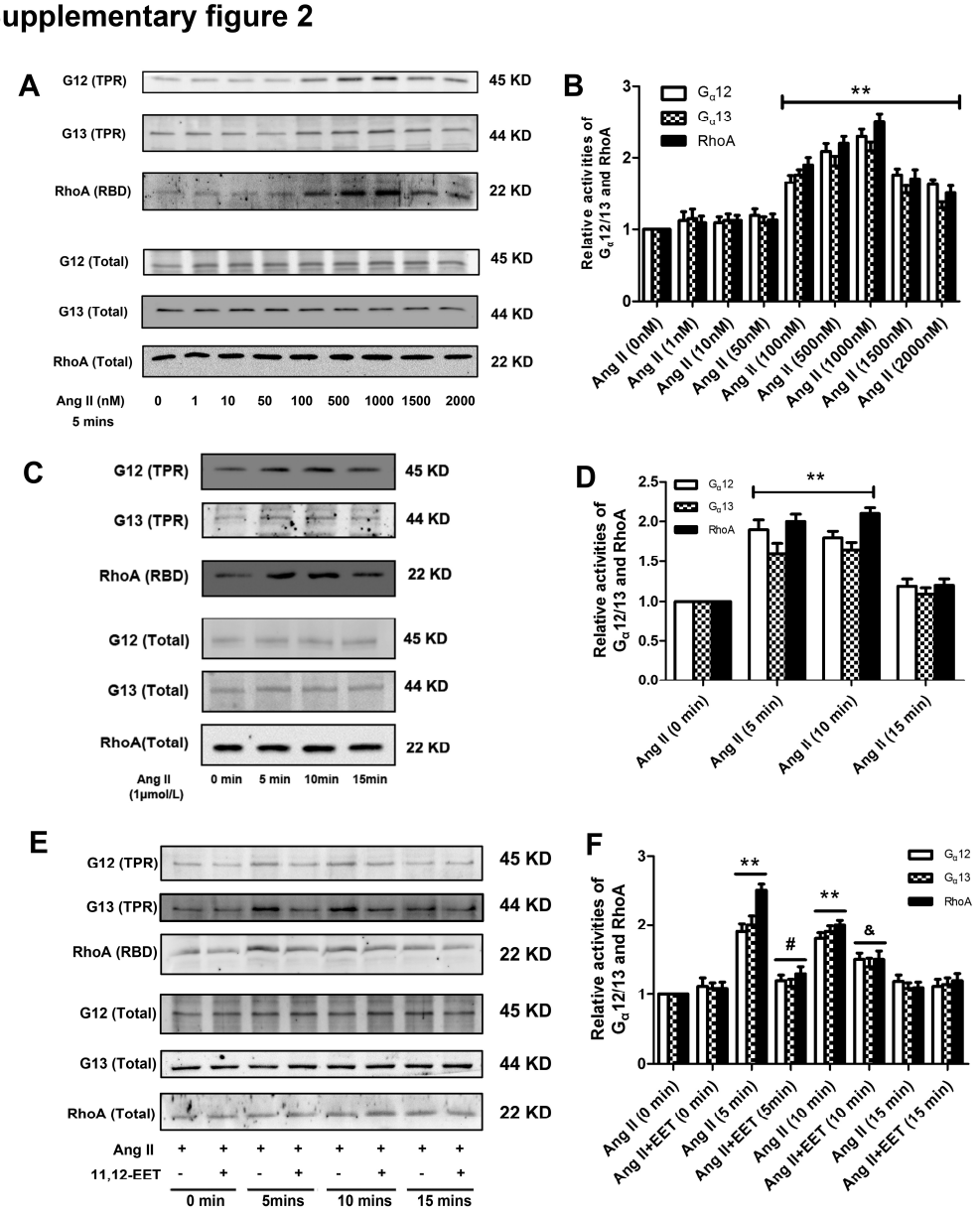
3. Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* **271**: 20246-20249.

Supplementary Figure 1. .Rationale of selecting 11,12-EET at concentration of 1μM for *in vitro* studies.



Supplementary Figure 1. Rationale of selecting 11,12-EET at concentration of 1μM for *in vitro* studies. (A) Representative Western blots and quantitation of COLI and α-SMA expression in cardiac fibroblasts treated with and without Ang II (1μM) or different EET regioisomer (1μM) for 24 hours. (B) Representative immunoblots and quantitation of α-SMA expression treated with and without Ang II (1μM) or different does of 11,12-EET (from 0.5nM to 1μM) for 24 hours. (n=5 for each experiment; **$p<0.05$ vs. DMSO; $^{\#}p<0.05$ vs. Ang-II)

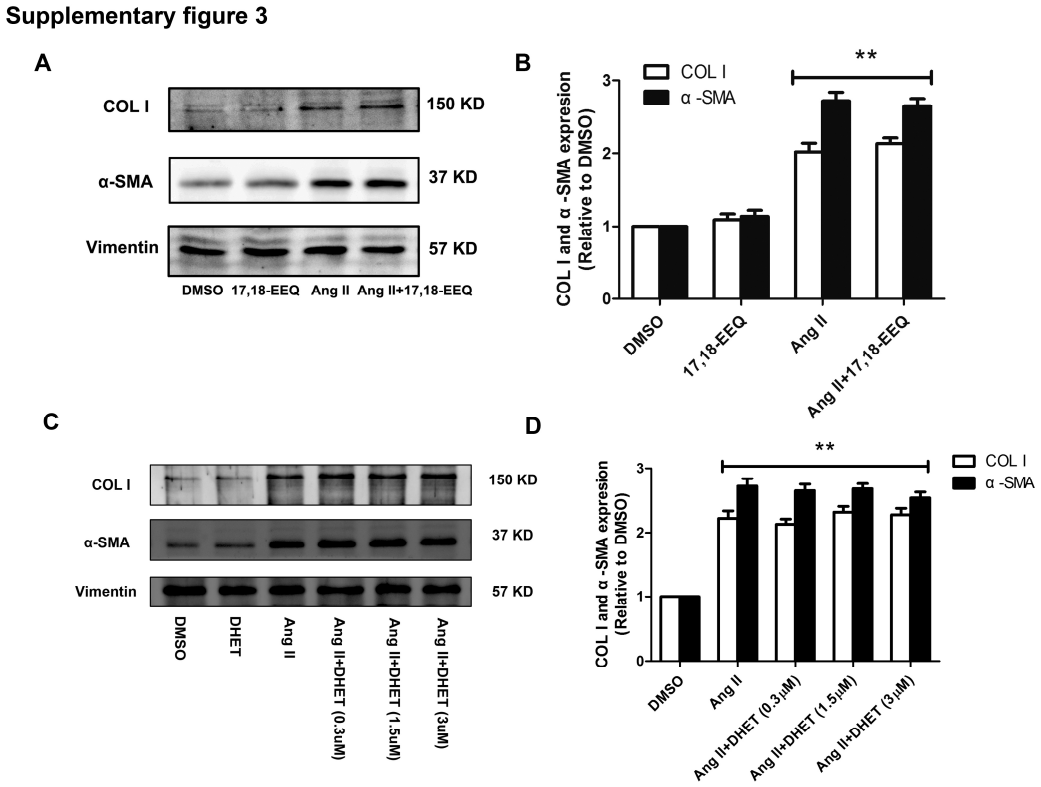
Supplementary Figure 2. Activation of $G\alpha_{12/13}$ and RhoA under various treatments



Supplementary Figure 2. Activation of $G\alpha_{12/13}$ and RhoA under various treatments (A) Representative immunoblots of G12 (TPR), G12 (total), G13 (TPR), G13 (total), RhoA (RBD) and RhoA (Total) in cardiac fibroblasts treated with various does of Ang II for 5 minutes. (B) Quantitation of $G\alpha_{12}$ activity, $G\alpha_{13}$ activity and RhoA activity by determine ratio of G12 (TPR)/G12 (Total), G13 (TPR)/G13 (Total) and RhoA (RBD)/RhoA (Total), respectively. (n=5 for each experiment; **p<0.05 vs. Ang-II-0nM) (C) Representative immunoblots of

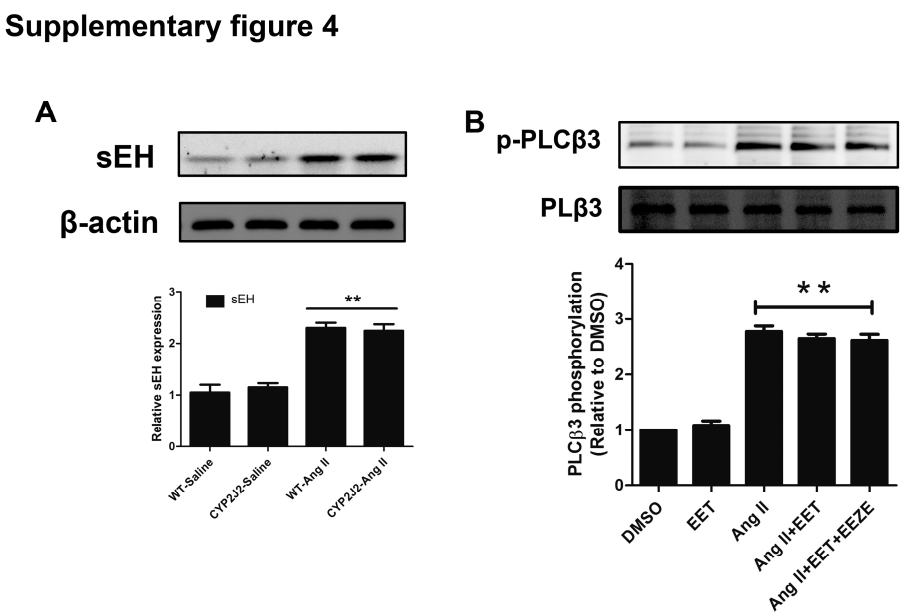
G12 (TPR), G12 (total), G13 (TPR), G13 (total), RhoA (RBD) and RhoA (Total) in cardiac fibroblasts treated with Ang II (1μM) for various incubation time. (D) Quantitation of $G\alpha_{12}$ activity, $G\alpha_{13}$ activity and RhoA activity by determine ratio of G12 (TPR)/G12 (Total), G13 (TPR)/G13 (Total) and RhoA (RBD)/RhoA (Total), respectively.（E）Representative immunoblots of G12 (TPR), G12 (total), G13 (TPR), G13 (total), RhoA (RBD) and RhoA (Total) in cardiac fibroblasts treated with and without Ang II (1μM) or 11,12-EET (1μM) for various incubation time. (F) Quantitation of $G\alpha_{12}$ activity, $G\alpha_{13}$ activity and RhoA activity by determine ratio of G12 (TPR)/G12 (Total), G13 (TPR)/G13 (Total) and RhoA (RBD)/RhoA (Total), respectively. (n=5 for each experiment; **$p<0.05$ vs. Ang-II-0min; $^{\#}p<0.05$ vs. Ang-II-5min; $^{\&}p<0.05$ vs. Ang-II-10min)

Supplementary Figure 3. 17,18-EEQ and 11,12-DHET could not prevent Ang II induced COLI and α-SMA upregulation.



Supplementary Figure 3. 17,18-EEQ and 11,12-DHET could not prevent Ang II induced COLI and α-SMA upregulation. (A) Representative Western blots of COLI and α-SMA in cardiac fibroblasts treated with and without Ang II (1μM) or 17,18-EEQ (1μM) for 24 hours. (B) Quantitation of COLI and α-SMA expression. (C) Representative Western blots of COLI and α-SMA in cardiac fibroblasts treated with and without Ang II (1μM) or various does of 11,12-DHET for 24 hours. (D) Quantitation of COLI and α-SMA expression. (n=5 for each experiment; **p<0.05 vs. DMSO)

Supplementary Figure 4. sEH upregulation and PLCβ3 phosphorylation were not inhibited by CYP2J2/EET.



Supplementary Figure 4. sEH upregulation and PLCβ3 phosphorylation were not inhibited by CYP2J2/EET. (A) Representative western blots and quantitation of sEH in cardiac tissue of various groups (n=10 for each group; **p<0.05 vs. WT-saline). (B) Representative Western blots and quantitation of PLCβ3 phosphorylation in cardiac fibroblasts treated with Ang II (1μM) in presence or absence of 11,12-EET (1μM) for 5min. (n=5 for each experiment; **p<0.05 vs. DMSO)