Published in final edited form as:
Circ Res. 2006 August 18; 99(4): 442–450.

Role of Soluble Epoxide Hydrolase in Postischemic Recovery of Heart Contractile Function

John M. Seubert*, **Christopher J. Sinal***, **Joan Graves**, **Laura M. DeGraff**, **J. Alyce Bradbury**, **Craig R. Lee**, **Kerry Goralski**, **Michelle A. Carey**, **Ayala Luria**, **John W. Newman**, **Bruce D. Hammock**, **John R. Falck**, **Holly Roberts**, **Howard A. Rockman**, **Elizabeth Murphy**, and **Darryl C. Zeldin**

From the Faculty of Pharmacy and Pharmaceutical Sciences (J.M.S.), University of Alberta, Edmonton, AB, Canada; the Division of Intramural Research (J.M.S., J.G., L.M.D., J.A.B., C.R.L., M.A.C., E.M., D.C.Z.), NIEHS/NIH, Research Triangle Park, NC; the Department of Pharmacology (C.J.S., K.G.), Dalhousie University, Halifax, NS, Canada; the Department of Entomology and Cancer Research Center (A.L., J.W.N., B.D.H.), University of California, Davis, Calif; the Departments of Biochemistry and Pharmacology (J.R.F.), University of Texas Southwestern Medical Center, Dallas, Tex; the Department of Medicine (H.R., H.A.R., D.C.Z.), Duke University Medical Center, Durham, NC; and the School of Pharmacy (C.R.L.), University of North Carolina at Chapel Hill, Chapel Hill, NC.

Abstract

Cytochrome P450 epoxygenases metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs) which are converted to dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (*Ephx2*, sEH). To examine the functional role of sEH in the heart, mice with targeted disruption of the *Ephx2* gene were studied. Hearts from sEH null mice have undetectable levels of sEH mRNA and protein and cannot convert EETs to DHETs. sEH null mice have normal heart anatomy and basal contractile function, but have higher fatty acid epoxide:diol ratios in plasma and cardiomyocyte cell culture media compared with wild type (WT). sEH null hearts have improved recovery of left ventricular developed pressure (LVDP) and less infarction compared with WT hearts after 20 minutes ischemia. Perfusion with the putative EET receptor antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (10 to 100 nmol/L) before ischemia abolishes this cardioprotective phenotype. Inhibitor studies demonstrate that perfusion with phosphatidylinositol-3 kinase (PI3K) inhibitors wortmannin (200 nmol/L) or LY294002 (5 μmol/L), the ATP-sensitive K^+ channel (K_{ATP}) inhibitor glibenclamide (1 μmol/L), the mitochondrial K_{ATP} (mitoK_{ATP}) inhibitor 5-hydroxydecanoate (100 to 200 μmol/L), or the Ca^{2+}-sensitive K^+ channel (K_{Ca}) inhibitor paxilline (10 μmol/L) abolishes the cardioprotection in sEH null hearts. Consistent with increased activation of the PI3K cascade, sEH null mice exhibit increased cardiac expression of glycogen synthase kinase-3β (GSK-3β) phospho-protein after ischemia. Together, these data suggest that targeted disruption of sEH increases the availability of cardioprotective EETs that work by activating PI3K signaling pathways and K^+ channels.

Correspondence to Darryl C. Zeldin, MD, National Institute of Environmental Health Sciences, 111 T.W. Alexander Drive, Research Triangle Park, NC 27709. E-mail zeldin@niehs.nih.gov
*Both authors contributed equally to this study.

Disclosures

D.C.Z. is a coinventor (with B.D.H.) on U.S. Patent no. 6 531 506 B1 (Inhibitors of Epoxide Hydrolases for the Treatment of Hypertension) which has been exclusively licensed to Arete Therapeutics, and is also a coinventor on U.S. Patent no. 6 916 843 B1 (Antiinflammatory Actions of Cytochrome P450 Epoxygenase-Derived Eicosanoids). B.D.H. received funding from NIEHS (significant), NIH (significant), and Arete Therapeutics (significant). B.D.H. is founder and stock owner of Arete Therapeutics and serves on the advisory board and management board of Arete Therapeutics. U.C. Davis owns stock in Arete Therapeutics.

Keywords

arachidonic acid; cytochrome P450; eicosanoid; ischemia/reperfusion

Arachidonic acid (AA), a polyunsaturated fatty acid normally found esterified to cell membrane glycero-phospholipids, can be released by phospholipases in response to ischemia.[1] Free AA is then available for metabolism by prostaglandin H2 synthases, lipoxygenases, and cytochrome P450 (CYP) monooxygenases to generate numerous metabolites, collectively termed eicosanoids.[1] CYP epoxygenases metabolize AA to 4 regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs), all of which are biologically active.[1] The actions of EETs are terminated by conversion to the corresponding and less biologically active dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases.[2] Two major epoxide hydrolases are found in mammalian tissues, the microsomal epoxide hydrolase (mEH) and the soluble epoxide hydrolase (sEH or *Ephx2*).[3] Previous work has demonstrated that sEH is the main enzyme involved in the in vivo hydrolysis of EETs.[4]

EETs are important components of many intracellular signaling pathways in both cardiac and extracardiac tissues. For example, EETs activate large conductance Ca^{2+}-sensitive K^+ channels (BK_{Ca}) in vascular smooth muscle cells resulting in hyperpolarization of the resting membrane potential and vasodilation of the coronary circulation.[5] This effect is diminished on hydrolysis of EETs to DHETs by sEH.[6] Other studies have shown that EETs display antiinflammatory, thrombolytic, and angiogenic properties within the vasculature.[7–9] In endothelial cells, EETs activate mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathways, increase intracellular cAMP levels, upregulate expression of nitric oxide synthase, and protect against hypoxia-reoxygenation injury.[8–10] In general, the effects of DHETs on these pathways are less pronounced.[7,9] Within the heart, EETs activate cardiac ATP-sensitive K^+ (K_{ATP}) channels,[11,12] enhance L-type calcium currents,[13] and improve postischemic recovery of left ventricular function.[12,14,15] Thus, alteration in the production or elimination of EETs may affect steady-state cellular levels of these bioactive eicosanoids in vivo and could potentially influence cardiac function.

Recently, we reported that transgenic mice with cardiac-specific overexpression of human CYP2J2 had increased cardiomyocyte EET biosynthesis and enhanced postischemic recovery of left ventricular function.[12] The mechanism for the cardioprotection involved activation of mitochondrial K_{ATP} channels and the p42/p44-MAPK pathway.[12] To further examine the cardiac effects of CYP-derived eicosanoids under basal conditions and after ischemia/reperfusion, and to determine the functional role of sEH in the heart, we evaluated mice with a targeted disruption of the *Ephx2* gene.[16] sEH null mice have normal heart anatomy and basal contractile function, but exhibit improved recovery of left ventricular function after global ischemia and less infarction. Moreover, our data suggest that this cardioprotection is mediated by CYP epoxygenase metabolites of AA and involves activation of the PI3K pathway and K^+ channels.

Materials and Methods

For an expanded Material and Methods section, see the online data supplement available at http://circres.ahajournals.org.

Animals

Mice with the targeted disruption of the *Ephx2* gene were obtained from Christopher Sinal (Dalhousie University, Halifax, NS, Canada).[16] These mice, backcrossed onto a C57BL6 genetic background for 5 generations, were used to rederive a new colony at NIEHS by embryo

transfer. All experiments used male and female mice aged 4 to 6 months, weighing 25 to 35g, and were approved by the NIEHS Animal Care and Use Committee.

Northern Analysis, Immunoblotting

Northern analysis and immunoblotting were as previously described.[12,16] Subcellular fractions were prepared from frozen mouse hearts. Immunoblots were probed with antibodies to sEH (generous gift from B.D.H.), GSK-3β, phospho-GSK-3β, p42/p44-MAPK, phospho-p42/p44-MAPK (Cell Signaling Technology, Inc, Beverly, Mass), BK_{Ca}-α, BK_{Ca}-β1, GAPDH, or actin C-11 (Santa Cruz Biotechnology, Santa Cruz, Calif).

Fatty Acid Metabolism

Heart cytosolic fractions (2 mg protein/mL) were incubated with [1-^{14}C]14,15-EET (100 μmol/L) as described.[17] Products were extracted and analyzed by high-performance liquid chromatography.[18] Epoxide hydrolase activity was calculated as the rate of 14,15-DHET produced/mg protein/min at 37°C. Neonatal cardiomyocyte cell culture media, adult plasma, and heart perfusate were analyzed for epoxy and dihydroxy fatty acid derivatives of AA and linoleic acid (LA) using established HPLC/MS/MS methods.[12,19]

Transthoracic Echocardiography, Assessment of Heart Anatomy

Two-dimensional guided M-mode echocardiography was performed using an HDI 5000 echocardiograph as described.[20] sEH null mice and WT littermate controls were then euthanized and hearts were removed, dissected, and weighed.

Isolated-Perfused Hearts

Hearts were perfused in the Langendorff mode as described.[12] Hearts were perfused with buffer for a 40-minute stabilization period then subjected to 20 minutes global no-flow ischemia, followed by 40 minutes reperfusion. For some experiments, hearts were stabilized for 20 minutes, then perfused with either the putative pan-EET receptor antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE, 10 to 100 nmol/L), 8,9-EET (1 μmol/L), 11,12-EET (1 μmol/L), 14,15-EET (1 μmol/L), the sarcolemmal K_{ATP} (sarcK_{ATP}) and mitochondrial K_{ATP} (mitoK_{ATP}) channel inhibitor glibenclamide (GLIB, 1 μmol/L), the selective mitoK_{ATP} channel inhibitor 5-hydroxydecanoate (5-HD, 100 to 200 μmol/L), the PI3K inhibitors wortmannin (200 nmol/L) or LY294002 (5 μmol/L), the p42/p44-MAPK kinase (MEK) inhibitor PD98059 (10 μmol/L), the BK_{Ca} channel inhibitor paxilline (10 μmol/L), or vehicle for 20 minutes, then subjected to 20 minutes ischemia and 40 minutes reperfusion in the presence of antagonist or inhibitors. Recovery of contractile function was taken as left ventricular developed pressure (LVDP) at the end of reperfusion expressed as a percentage of preischemic LVDP. To determine the amount of infarction, after 2 hour reperfusion, hearts were incubated with a 1% solution of 2,3,5-triphenyl-tetrazolium chloride (TTC) dissolved in Krebs–Henseleit buffer at 37°C for 10 minutes, then fixed in formalin and cut into thin cross-sectional slices. The area of infarction was quantified by measuring stained (red, live tissue) and unstained (white, necrotic) regions. LDH activity was assessed in heart perfusates using a commercially available assay (Roche Applied Sciences).

Statistical Analysis

Values are expressed as mean ± SEM. Data were analyzed by ANOVA or Student *t* test using SYSTAT software (SYSTAT Inc). Values were considered significantly different if $P<0.05$.

Results

Initial Characterization of sEH Null Hearts

Northern analysis with the sEH cDNA probe (Figure 1a) and immunoblotting with an sEH-specific antibody (Figure 1b) revealed that sEH null hearts lacked expression of sEH at both the mRNA and protein levels. Cytosolic fractions from WT hearts rapidly converted 14,15-EET to 14,15-DHET (rate: 9.6 ± 0.6 nmol DHET formed/mg cytosolic protein/min; Figure 1c). In contrast, cytosolic fractions from sEH null hearts were unable to convert 14,15-EET to 14,15-DHET (Figure 1d), indicating reduced capacity for EET hydrolysis. To further assess fatty acid metabolism from endogenous lipid pools, we measured levels of CYP epoxygenase-derived products of AA and LA in plasma and cardiomyocyte cell culture media from sEH null and WT mice by HPLC/MS/MS. The sEH null mice had elevated plasma levels of fatty acid epoxides and depressed plasma levels of fatty acid diols relative to WT controls (see online supplement). Thus, the ratio of plasma EETs to DHETs was higher in sEH null compared with WT mice (Figure 1e). Similarly, the ratio of plasma epoxyoctadecenoic acids (EpOME) to dihydroxyocta-decenoic acids (DHOMEs) was higher in sEH null mice relative to WT controls (Figure 1e). Similar results were obtained for cardiomyocyte cell culture media (Figure 1f and online supplement). Levels of EETs and DHETs in cardiac perfusates before and after ischemia in both WT and sEH null mice were at or below detection limits of the HPLC/MS/MS assay, and therefore could not be quantified. Taken together, these data indicate that sEH null mice exhibit altered fatty acid epoxide metabolism in cardiomyocytes in vitro and in the systematic circulation in vivo.

Heart Anatomy and Baseline Function

The Table summarizes anatomic and functional characteristics in sEH null and WT hearts. There were no significant differences between the two groups in heart or individual chamber weights, echocardiographic dimensions, or fractional shortening, heart rate, or hemodynamic parameters under basal conditions. These data indicate that sEH null hearts are anatomically and functionally normal at baseline.

Cardiac Performance After Ischemia/Reperfusion in Perfused Hearts

Isolated-perfused sEH null hearts had normal baseline contractile function, measured as LVDP (Figure 2a; Table). Hearts from sEH null mice had significantly improved postischemic recovery of LVDP compared with hearts from WT littermate controls. The improved function was evident within 10 minutes of reperfusion and persisted throughout the recovery period (Figure 2a). At 40 minutes reflow, LVDP recovery was significantly higher in sEH null (51 ± 3%) than WT hearts (22 ± 2%) (Figure 2b and 2c; Table). There were no differences between sEH null and WT hearts in time-to-onset of ischemic contracture (16.0 ± 0.7 minutes versus 15.0 ± 0.5 minutes, $P = 0.15$) or maximal ischemic contracture (73 ± 4 cm H_2O versus 70 ± 6 cm H_2O, $P = 0.55$). After 20 minutes of global ischemia and 120 minutes of reperfusion, sEH null hearts had significantly less infarction than WT hearts (Figure 2d and 2e). Consistent with these results, there was a significant reduction in LDH activity in sEH null heart perfusate compared with WT during reperfusion (Figure 2f).

To determine whether the improved postischemic functional recovery in sEH null hearts was mediated by CYP epoxygenase metabolites, we conducted experiments in the presence of 14,15-EEZE. This putative pan-EET receptor antagonist caused a small reduction in postischemic LVDP recovery in WT mice at high concentrations (Figure 2c). Importantly, 14,15-EEZE completely abolished the improved postischemic recovery of LVDP in sEH null mice; recovery of LVDP at 40 minutes reflow was 52 ± 5% in the absence of 14,15-EEZE, 23 ± 4% in the presence of 10 nmol/L 14,15-EEZE, and 16 ± 3% in the presence of 100 nmol/L 14,15-EEZE (Figure 2c). Thus, percent LVDP recovery was comparable in the two genotypes

after treatment with 14,15-EEZE. Moreover, perfusion of WT hearts with physiologically relevant concentrations (1 μmol/L) of 8,9-EET, 11,12-EET, or 14,15-EET resulted in improved postischemic functional recovery compared with perfusion with vehicle (LVDP = 33.6 ± 8.5%, 36.5 ± 5.2%, 27.6 ± 1.6%, and 20.4 ± 1.0%, respectively, n = 4 to 8 per group, $P<0.05$ versus vehicle for each EET regioisomer). Together, these data indicate that the cardioprotective phenotype in sEH null mice is mediated by CYP epoxygenase metabolites, presumably EETs.

Role of MAPK and PI3K Pathways in Postischemic Functional Recovery

To determine whether the MAPK signaling pathway was involved in the cardioprotective mechanism, we examined the phosphorylation status of p42/p44-MAPK in WT and she null hearts and examined the effect of the MEK inhibitor PD98059 on postischemic functional recovery. We did not observe differences between sEH null and WT mice in phosphorylation of p42/p44-MAPK (Figure 3a). Moreover, perfusion with PD98059 did not block the improved functional recovery in sEH null hearts (Figure 3b). Together, these data indicate that the MAPK pathway is not involved in the cardioprotective phenotype of sEH null mice.

To determine the role of the PI3K signaling cascade in the cardioprotective response, we conducted experiments in the presence or absence of the PI3K inhibitors wortmannin or LY294002. Neither inhibitor had a significant effect on baseline LVDP in our model system (data not shown). Perfusion with either wortmannin or LY294002 for 20 minutes before ischemia had no effect on postischemic LVDP recovery in WT hearts; however, both inhibitors completely abolished the improved postischemic functional recovery in sEH null hearts (Figure 4a). Thus, percent LVDP recovery at 40 minutes reperfusion was comparable in the two genotypes after treatment with either wortmannin or LY294002 (Figure 4a). These data suggest the involvement of the PI3K cascade in the cardioprotective phenotype observed in sEH null mice.

To further assess downstream targets in the PI3K cascade, we examined the phosphorylation status of GSK-3β in WT and sEH null hearts at baseline, during ischemia, and during reperfusion. There were no significant differences in the expression of phospho–GSK-3β between WT and sEH null hearts under basal conditions (Figure 4b). Interestingly, levels of phospho–GSK-3β were significantly higher in sEH null hearts compared with WT hearts at 5 minutes of reperfusion (Figure 4b). Likewise, the ratio of phospho–GSK-3β to total GSK-3β expression was significantly greater in sEH null hearts than in WT hearts during early reperfusion (Figure 4d). These differences were no longer present during late reperfusion. The increased phosphorylation of GSK-3β during early reperfusion in sEH null mice was significantly attenuated by the PI3K inhibitor wortmannin (200 nmol/L; Figure 4c and 4d). Together with recent data on the central role of GSK-3β activation in cardioprotection,[21] these data suggest one possible mechanism for the observed postischemic recovery in sEH null mice.

Role of K^+ Channels in Postischemic Functional Recovery

To determine whether K_{ATP} channels were involved in the cardioprotective mechanism, we conducted experiments in the presence or absence of the sarcK_{ATP} and mitoK_{ATP} inhibitor GLIB or the selective mitoK_{ATP} inhibitor 5-HD. Neither GLIB nor 5-HD had significant effects on baseline LVDP (data not shown). Perfusion with either GLIB or 5-HD for 20 minutes before ischemia completely abolished the improved postischemic functional recovery in sEH null hearts (Figure 5a). Thus, percent LVDP recovery at 40 minutes reperfusion was comparable in the two genotypes after treatment with either GLIB or 5-HD (Figure 5a).

To examine the potential role of BK_{Ca} channels in the cardioprotective phenotype, we examined the effects of the specific BK_{Ca} inhibitor paxilline (100 μmol/L) on postischemic LVDP. Administration of paxilline abolished the improved postischemic functional recovery

in sEH null hearts (Figure 5b). There were no differences in expression of the BK_{Ca}-β1 subunit between WT and sEH null hearts (Figure 5c). Expression of the BK_{Ca}-α subunit was low-undetectable in both WT and sEH null hearts, consistent with a recent publication.[22]

Discussion

Cardiac CYP epoxygenases convert AA to biologically active EETs that have well established effects within the cardiovascular system.[12,18] The activity of these compounds is terminated mainly through conversion to less active DHETs by sEH.[2] Recent studies demonstrate that CYP epoxygenase-derived eicosanoids can affect cardiomyocyte function[11,13,14] and improve postischemic LVDP recovery.[12,14,15] In addition, EET biosynthesis is enhanced in stenosed coronary arteries[23] and during cardiac ischemia/reperfusion injury.[24] The data presented herein provide further evidence that EETs are involved in the cardioprotective response. We demonstrate, for the first time, that targeted disruption of the *Ephx2* gene leads to improved recovery of left ventricular function and reduced infarction after ischemia. Moreover, the putative EET receptor antagonist 14,15-EEZE abolishes the cardio-protection in these mice specifically implicating EETs in this process. Taken together, these data suggest that EETs have important functional effects in the ischemic heart and that augmentation of EET levels by inhibition of sEH may represent a novel approach to the treatment of ischemic heart disease.

Heart anatomy and basal contractile function are similar in sEH null and WT mice, suggesting that disruption of the *Ephx2* gene has no detrimental cardiac effects under normal conditions. However, sEH null mice have altered fatty acid metabolism compared with WT mice at baseline. Indeed, increased LA and AA epoxide:diol ratios were found in the circulation and in cardiomyocyte cell culture media from sEH null mice. Moreover, conversion of 14,15-EET to 14,15-DHET was absent in heart cytosolic fractions from sEH null mice. The majority of endogenous EETs (>85%) are esterified to membrane glycerophospoholipids where they are generally considered inactive until their release.[25] The cardiac effects of sEH disruption occurred only after ischemia, presumably attributable to enhanced release of EETs from phospholipid stores. In this regard, ischemia has been shown to activate cytosolic phospholipase A_2, leading to the release of bioactive eicosanoids from glycerophospholipids.[26] The results in the current article are consistent with our recent findings that mice overexpressing human CYP2J2 in the heart have normal heart anatomy and function at baseline, but have elevated cardiomyocyte EET levels and improved postischemic functional recovery.[12]

AA can be metabolized by CYP epoxygenases and CYP ω-hydroxylases to products that have different physiologic effects. For example, EETs have potent vasodilatory properties and 20-HETE has vasoconstrictive effects.[1] Changes in expression or activity of specific CYP enzymes can alter the delicate balance between EETs and 20-HETE. For example, recent data demonstrates that inhibition of CYP ω-hydroxylases results in reduction of infarction after ischemic injury, suggesting that 20-HETE has detrimental effects within the heart.[27,28] These studies highlight the complexity of the CYP enzyme system and emphasize the role of different CYP metabolites in cardioprotection. Many CYP inhibitors lack isoform specificity and also may have effects in other signaling pathways. By targeting sEH, our study can more directly address the role of CYP epoxygenase products in the cardiovascular system and minimize ambiguity in deciphering the relative importance of individual enzymes and their metabolites.

The sEH null hearts have reduced levels of LA diols (DHOMEs) which have been shown to cause adverse cardiac effects, such as mitochondrial dysfunction[29] and altered cardiac electrical activity.[30] Thus, increased EETs and decreased DHOMEs are beneficial to the cardiovascular system and could both be responsible for the observed cardioprotection in sEH

null mice. To determine the relative importance of EETs in this model, we used 14,15-EEZE, which has been shown to be selective for inhibition of EET effects within the cardiovascular system.[31] At the highest dose, treatment with 14,15-EEZE caused a small reduction in postischemic LVDP recovery in WT mouse hearts consistent with a role for EETs in this process. Importantly, 14,15-EEZE abolished the improved postischemic LVDP recovery in sEH null mouse hearts, indicating that the cardioprotective effects were mediated by EETs. Although it is possible that 14,15-EEZE has effects that are unrelated to EET antagonism,[32] these results are consistent with both current and previous data demonstrating that addition of EETs to heart perfusates before ischemia results in improved postischemic functional recovery in both rats and mice.[12,14]

Hydrolysis of EETs by sEH is dependent on both regio-and stereoselective parameters.[33] 14,15-EET is the preferred sEH substrate, and 5,6-EET is an extremely poor substrate for this enzyme.[33] The lipid metabolite profiles observed in cardiomyocyte cultures in sEH null mice showed altered epoxide:diol ratios for the 8,9-, 11,12-, and 14,15-EET regioisomers, but not for the 5,6-EET regioisomer. These data are consistent with the known substrate preferences of sEH.

The lack of difference in p42/p44-MAPK activation between sEH null and WT mice is in sharp contrast to our previous study, where phosphorylation of p42/p44 was enhanced in CYP2J2 transgenic mice and administration of PD98059 completely blocked the improved function recovery.[12] This suggests that different mechanisms may underlie the cardioprotection in sEH null and CYP2J2 transgenic mice, both of which have increased cardiac EETs. Possible reasons for these differences include: (1) CYP2J2 overexpression was restricted to cardiomyocytes in the transgenic mice whereas sEH disruption occurred in both cardiomyocytes and endothelial cells; and (2) DHETs are reduced in sEH null mice but increased in CYP2J2 transgenic mice.

Activation of the PI3K signaling pathway targets multiple cellular functions, including survival, proliferation, and vesicle trafficking.[34] The PI3K signaling pathway has been shown to be involved in cardioprotective responses after ischemia/reperfusion.[35,36] Signaling through PI3K promotes a prosurvival mechanism involving recruitment of downstream kinases such as GSK-3β.[34,35] The fact that two structurally different PI3K inhibitors attenuate the improved postischemic LVDP recovery in sEH null mice provides strong evidence for enhanced activation of this pathway. In this regard, we observed increased phospho–GSK-3β expression during early reperfusion in sEH null hearts, a phenomenon which was abolished by PI3K inhibition. Consistent with these data, cell culture studies have demonstrated that EETs activate the PI3K-Akt pathway.[8] Recent evidence suggests that convergence of multiple cardioprotective signaling pathways onto GSK-3β may represent an integrated mechanism of cellular protection.[21] It was purposed that this convergence targets the mitochondrial permeability transition pore (MPTP) complex limiting its opening.[21] Inhibition of MPTP induction early during reperfusion has cardioprotective effects.[35]

A large body of research suggests a role for K_{ATP} channels in cardioprotection.[37] Cardiomyocyte studies have demonstrated that EETs enhance both sarcK_{ATP} and mitoK_{ATP} channel activities.[11,12] In the current study, administration of either the sarcK_{ATP} channel inhibitor glibenclamide or the mitoK_{ATP} channel inhibitor 5-HD attenuated the improved postischemic recovery of sEH null hearts. The precise identity of the mitoK_{ATP} channel has yet to be determined, therefore it is unknown whether EETs directly target these channels or work through upstream signals such as PI3K. Recent work has suggested the role of BK_{Ca} channels in protection against ischemia/reperfusion injury.[38] EETs are known activators and ω-HETEs are known inhibitors of these channels in vascular smooth muscle cells.[1,5] Newly identified K_{Ca} channels in cardiac mitochondria (mitoK_{Ca}) are thought to work in concert with mitoK_{ATP} and other mitochondrial proteins to mediate cardioprotection.[38,39] Our experiments

demonstrate that the BK_{Ca} channel inhibitor paxilline abolished the improved postischemic functional recovery in sEH hearts, suggesting a role for this channel in the cardioprotective mechanism. Activation of the channels might protect the myocardium by increasing mitochondrial K^+ uptake and, in turn, reducing Ca^{2+} overload, with a net effect of protecting mitochondrial function.[38,39]

We postulate that the enhanced functional recovery observed in the sEH null mice entails modulation of both PI3K and mitochondrial K^+ channels. Figure 6 shows a schematic of the proposed mechanisms whereby disruption of sEH leads to cardioprotection via increasing EET levels.

Recent epidemiologic data suggests an association between polymorphisms in the genes encoding *CYP2J2*, *CYP2C8*, *CYP2C9*, and *EPHX2* and cardiovascular disease risk in humans suggesting the relevance of the CYP epoxygenase pathway in the heart.[40–42] The data in the current manuscript is consistent with an important modulatory effect of CYP-derived eicosanoids in general and sEH in particular in cardioprotection. The present study demonstrates that targeted disruption of *Ephx2* in mice has no effect on basal heart anatomy and function but results in improved postischemic recovery of LVDP. Taken together, these data suggest that manipulating sEH activity may represent a novel therapeutic approach to management of ischemic heart disease in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Drs Thomas Eling and Robert Langenbach for helpful suggestions.

Source of Funding

This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (to D.C.Z.), NIH ES04699 (to B.D.H.), and Canadian Institutes of Health Research (to J.M.S and C.J.S.). C.R.L. is the recipient of a NIH graduate fellowship (ES012856).

References

1. Roman RJ. P450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol Rev 2002;82:131–185. [PubMed: 11773611]
2. Morisseau C, Hammock BD. Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. Annu Rev Pharmacol Toxicol 2005;45:311–333. [PubMed: 15822179]
3. Fang X, Weintraub NL, McCaw RB, Hu S, Harmon SD, Rice JB, Hammock BD, Spector AA. Effect of soluble epoxide hydrolase inhibition on epoxyeicosatrienoic acid metabolism in human blood vessels. Am J Physiol Heart Circ Physiol 2004;287:H2412–H2420. [PubMed: 15284062]
4. Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, Capdevila JH. Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. J Biol Chem 1993;268:6402–6407. [PubMed: 8454612]
5. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ Res 1996;78:415–423. [PubMed: 8593700]
6. Fang X, Kaduce TL, Weintraub NL, Harmon S, Teesch LM, Morisseau C, Thompson DA, Hammock BD, Spector AA. Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition. J Biol Chem 2001;276:14867–14874. [PubMed: 11278979]
7. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 1999;285:1276–1279. [PubMed: 10455056]

8. Wang Y, Wei X, Xiao X, Hui R, Card JW, Carey MA, Wang DW, Zeldin DC. Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via MAPK and PI3K/Akt signaling pathways. J Pharmacol Exp Ther 2005;314:522–532. [PubMed: 15840765]
9. Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, Liao JK. Activation of $G\alpha_s$ mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. J Biol Chem 2001;276:15983–15989. [PubMed: 11279071]
10. Yang B, Graham L, Dikalov S, Mason RP, Falck JR, Liao JK, Zeldin DC. Overexpression of cytochrome P450 CYP2J2 protects against hypoxiareoxygenation injury in cultured bovine aortic endothelial cells. Mol Pharmacol 2001;60:310–320. [PubMed: 11455018]
11. Lee HC, Lu T, Weintraub NL, VanRollins M, Spector AA, Shibata EF. Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes. J Physiol 1999;519:153–168. [PubMed: 10432346]
12. Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman J, Mao L, Rockman HA, Hammock BD, Murphy E, Zeldin DC. Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K^+ channels and p42/p44-MAPK pathway. Circ Res 2004;95:506–514. [PubMed: 15256482]
13. Xiao YF, Huang L, Morgan JP. Cytochrome P450: A novel system modulating Ca^{2+} channels and contraction in mammalian heart cells. J Physiol 1998;508:777–792. [PubMed: 9518732]
14. Wu S, Chen W, Murphy E, Gabel S, Tomer KB, Foley J, Steenbergen C, Falck JR, Moomaw CR, Zeldin DC. Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. J Biol Chem 1997;272:12551–12559. [PubMed: 9139707]
15. Nithipatikom K, Moore JM, Isbell MA, Falck JR, Gross GJ. Epoxyeicosatrienoic acids in cardioprotection: ischemic versus reperfusion injury. Am J Physiol Heart Circ Physiol 2006;29:H537–H542. [PubMed: 16473964]
16. Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, Gonzalez FJ. Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. J Biol Chem 2000;275:40504–40510. [PubMed: 11001943]
17. Gulick J, Subramaniam A, Neumann J, Robbins J. Isolation and characterization of the mouse cardiac myosin heavy chain genes. J Biol Chem 1991;266:9180–9185. [PubMed: 2026617]
18. Wu S, Moomaw CR, Tomer KB, Falck JR, Zeldin DC. Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. J Biol Chem 1996;271:3460–3468. [PubMed: 8631948]
19. Newman JW, Watanabe T, Hammock BD. The simultaneous quantification of cytochrome P450 dependent linoleate and arachidonate metabolites in urine by HPLC/MS/MS. J Lipid Res 2002;43:1563–1578. [PubMed: 12235189]
20. Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ, Rockman HA. Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. Circulation 2002;105:85–92. [PubMed: 11772881]
21. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ. Glycogen synthase kinase-3β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004;113:1535–1549. [PubMed: 15173880]
22. Ohya S, Kuwata Y, Sakamoto K, Muraki K, Imaizumi Y. Cardioprotective effects of estradiol include the activation of large-conductance Ca^{2+}-activated K^+ channels in cardiac mitochondria. Am J Physiol Heart Circ Physiol 2005;289:H1635–H1642. [PubMed: 16113069]
23. Rosolowsky M, Falck JR, Willerson JT, Campbell WB. Synthesis of lipoxygenase and epoxygenase products of arachidonic acid by normal and stenosed canine coronary arteries. Circ Res 1990;66:608–621. [PubMed: 2106399]
24. Nithipatikom K, DiCamelli RF, Kohler S, Gumina RJ, Falck JR, Campbell WB, Gross GJ. Determination of cytochrome P450 metabolites of arachidonic acid in coronary venous plasma during ischemia and reperfusion in dogs. Anal Biochem 2001;292:115–124. [PubMed: 11319825]
25. Spector AA, Fang X, Snyder GD, Weintraub NL. Epoxyeicosatrienoic acids: metabolism and biochemical function. Prog Lipid Res 2004;43:55–90. [PubMed: 14636671]

26. Saluja I, Song D, O'Regan MH, Phillis JW. Role of phospholipase A_2 in the release of free fatty acids during ischemia-reperfusion in the rat cerebral cortex. Neurosci Lett 1997;233:97–100. [PubMed: 9350841]
27. Gross ER, Nithipatikom K, Hsu AK, Peart JN, Falck JR, Campbell WB, Gross GJ. Cytochrome P450 omega-hydroxylase inhibition reduces infarct size during reperfusion via the sarcolemmal K_{ATP} channel. J Mol Cell Cardiol 2004;37:1245–1249. [PubMed: 15572055]
28. Nithipatikom K, Endsley MP, Moore JM, Isbell MA, Falck JR, Campbell WB, Gross GJ. Effects of selective inhibition of cytochrome P450 omega-hydroxylases and ischemic preconditioning in myocardial protection. Am J Physiol Heart Circ Physiol 2006;290:H500–H505. [PubMed: 16214838]
29. Sisemore MF, Zheng J, Yang JC, Thompson DA, Plopper CG, Cortopassi GA, Hammock BD. Cellular characterization of leukotoxin diol-induced mitochondrial dysfunction. Arch Biochem Biophys 2001;392:32–37. [PubMed: 11469791]
30. Stimers JR, Dobretsov M, Hastings SL, Jude AR, Grant DF. Effects of linoleic acid metabolites on electrical activity in adult rat ventricular myocytes. Biochim Biophys Acta 1999;1438:359–368. [PubMed: 10366778]
31. Gauthier KM, Falck JR, Reddy LM, Campbell WB. 14,15-EET analogs: characterization of structural requirements for agonist and antagonist activity in bovine coronary arteries. Pharmacol Res 2004;49:515–524. [PubMed: 15026029]
32. Harrington LS, Falck JR, Mitchell JA. Not so eeze: The 'EDHF' antagonist 14,15-epoxyeicosa-5(z)-enoic acid has vasodilator properties in mesenteric arteries. Eur J Pharmacol 2004;506:165–168. [PubMed: 15588737]
33. Zeldin DC, Wei S, Falck JR, Hammock BD, Snapper JR, Capdevila JH. Metabolism of epoxyeicosatrienoic acids by cytosolic epoxide hydrolase: substrate structural determinants of asymmetric catalysis. Arch Biochem Biophys 1995;316:443–451. [PubMed: 7840649]
34. Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH. The role of PI3K and PTEN in cardiovascular physiology and disease. J Mol Cell Cardiol 2004;37:449–471. [PubMed: 15276015]
35. Hausenloy D, Wynne A, Duchen M, Yellon D. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. Circulation 2004;109:1714–1717. [PubMed: 15066952]
36. Tong H, Chen W, London RE, Murphy E, Steenbergen C. Preconditioning enhanced glucose uptake is mediated by p38-MAPK not by PI3K. J Biol Chem 2000;275:11981–11986. [PubMed: 10766828]
37. Liu Y, Sato T, O'Rourke B, Marban E. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? Circulation 1998;97:2463–2469. [PubMed: 9641699]
38. Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, O'Rourke B. Cytoprotective role of Ca^{2+}-activated K^+ channels in the cardiac inner mitochondrial membrane. Science 2002;298:1029–1033. [PubMed: 12411707]
39. Hanley PJ, Daut J. K_{ATP} channels and preconditioning: a re-examination of the role of mitochondrial K_{ATP} channels and an overview of alternative mechanisms. J Mol Cell Cardiol 2005;39:17–50. [PubMed: 15907927]
40. Spiecker M, Darius H, Hankeln T, Soufi M, Sattler AM, Schaefer JR, Node K, Borgel J, Mugge A, Lindpaintner K, Huesing A, Maisch B, Zeldin DC, Liao JK. Risk of coronary artery disease associated with polymorphism of the cytochrome P450 epoxygenase CYP2J2. Circulation 2004;110:2132–2136. [PubMed: 15466638]
41. Yasar U, Bennet AM, Eliasson E, Lundgren S, Wiman B, De Faire U, Rane A. Allelic variants of cytochromes P450 2C modify the risk for acute myocardial infarction. Pharmacogenetics 2003;13:715–720. [PubMed: 14646690]
42. Lee CR, North KE, Bray MS, Fornage M, Seubert JM, Newman JW, Hammock BD, Couper DJ, Heiss G, Zeldin DC. Genetic variation in soluble epoxide hydrolase (EPHX2) and risk of coronary heart disease: the Atherosclerosis Risk in Communities study. Hum Mol Genet 2006;15:1640–1649. [PubMed: 16595607]

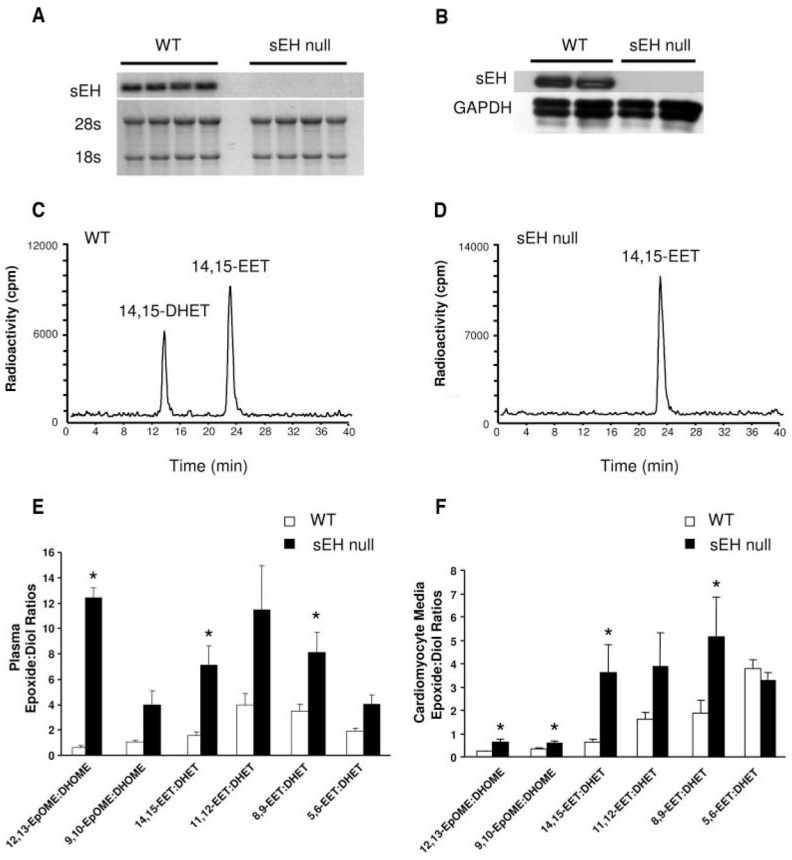

Figure 1.

Identification and initial characterization of sEH null mice. a, Northern blot showing absence of sEH transcripts in sEH null heart RNA, n=4. b, Immunoblots demonstrating lack of sEH protein expression in hearts of sEH null mice and abundant expression in WT mice. c and d, Representative HPLC chromatograms showing 14,15-EET metabolism to 14,15-DHET by cytosolic fractions prepared from hearts of WT (c) and sEH null (d) mice. e, Ratio of arachidonic acid (EET:DHET) and linoleic acid (EpOME:DHOME) epoxides to diols in plasma from sEH null and WT mice. f, Ratio of arachidonic acid and linoleic acid epoxides to diols in cardiomyocyte cell culture media from sEH null and WT mice. Values shown are mean ± SEM; n=4 to 5 per group; **P*<0.05 vs WT.

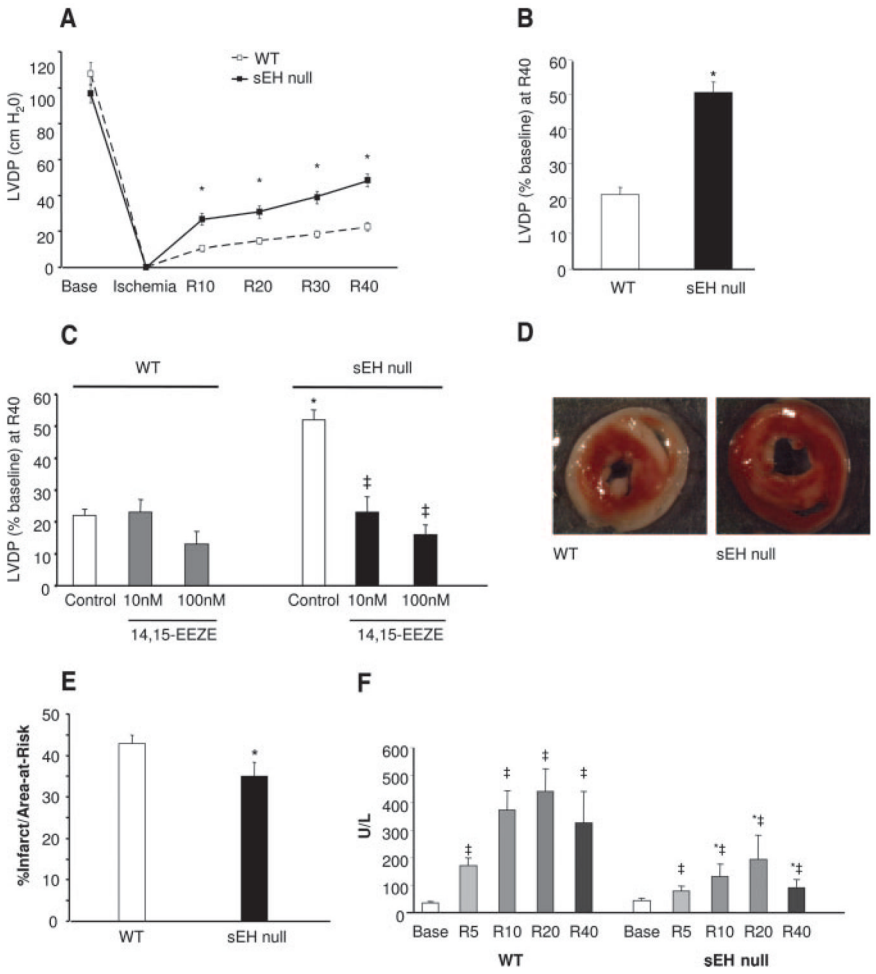

Figure 2.

Postischemic recovery of left ventricular function and infarction in sEH null and WT mice. a, LVDP in sEH null and WT mice. Values shown are mean ± SEM; n = 28 to 42; **P*<0.05 vs WT. b, Recovery of LVDP at 40 minutes reperfusion expressed as a percentage of baseline LVDP in hearts from sEH null and WT mice. c, Effects of the putative EET receptor antagonist 14,15-EEZE on postischemic recovery of LVDP in WT and sEH null hearts. Values represent mean ± SEM; n = 4 to 12; ‡*P*<0.05 vs vehicle control of same genotype; **P*<0.05 vs WT. d, Representative pictures of TTC staining in sEH null and WT hearts. TTC stains surviving tissues red and leaves infarcted areas white. e, Quantification of infarct size. Values represent mean ± SEM; n = 5; **P*<0.05 vs WT. f, LDH activity in perfusates from WT and sEH null hearts. Values represent mean ± SEM; n = 6 to 12 per group; ‡*P*<0.05 vs baseline of same genotype; **P*<0.05 vs WT at same time point.

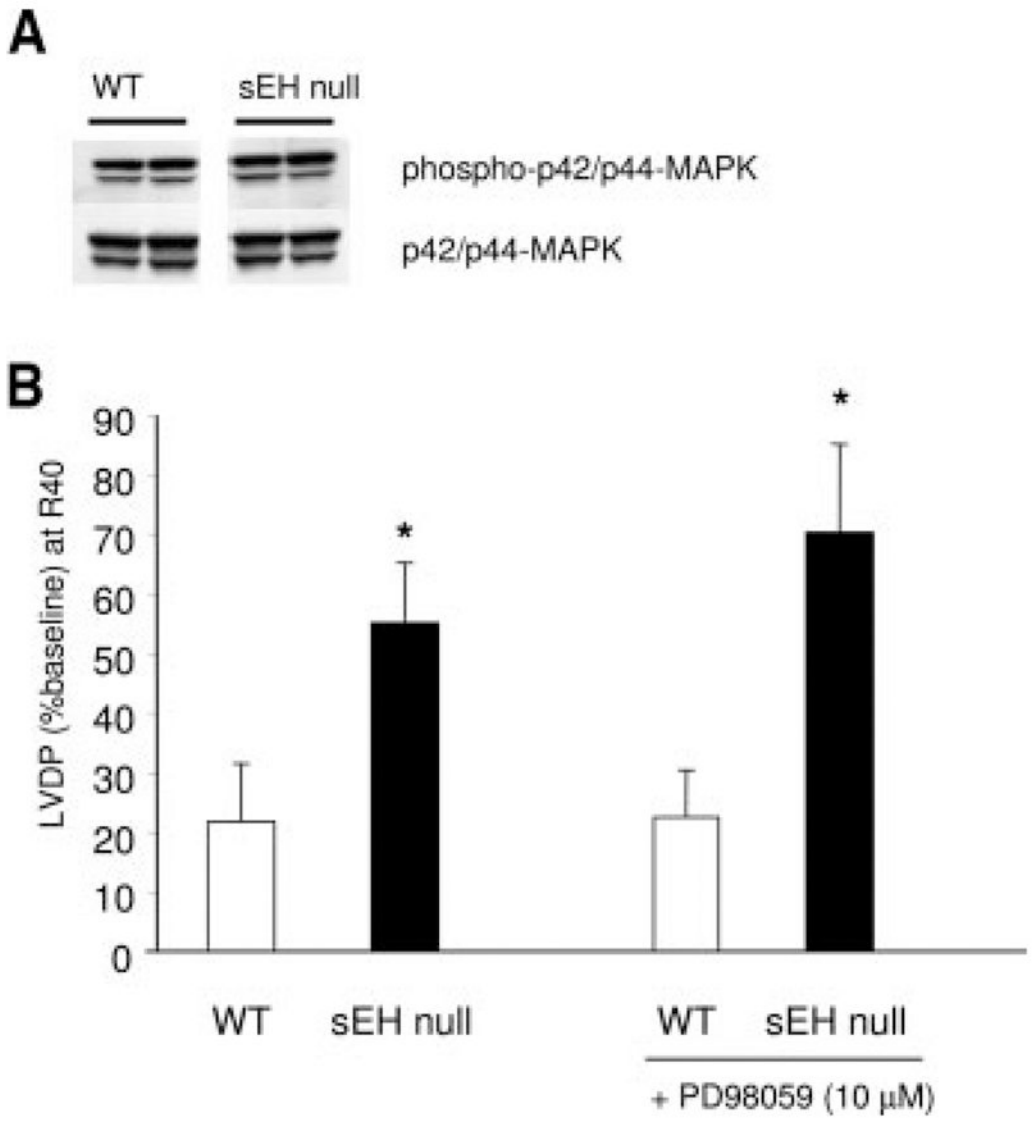

Figure 3.

Activation status of p42/p44-MAPK in WT and sEH null hearts. a, Representative immunoblots showing expression of phospho-p42/p44-MAPK and total p42/p44-MAPK in hearts after 5 minutes reperfusion. Similar results were obtained at other time points. b, Effects of MEK inhibition on postischemic recovery of LVDP in WT and sEH null hearts. Hearts were administered vehicle or PD98059 (10 μmol/L) before ischemia. Values represent mean ± SEM; n = 4 to 6 per group; *P<0.05 vs WT.

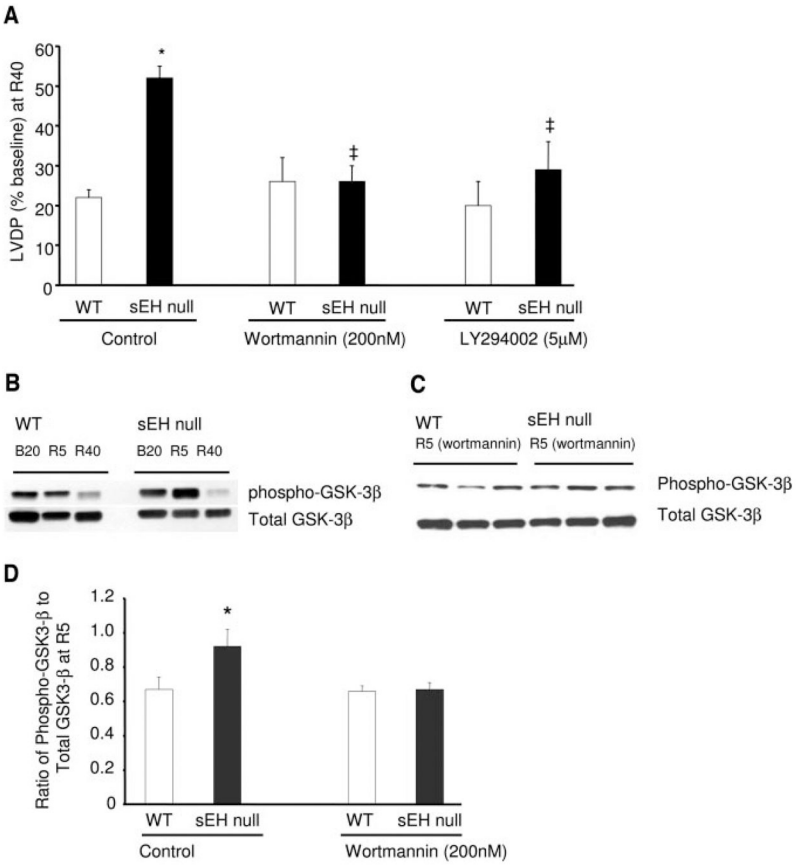

Figure 4.

Role of PI3K and GSK-3*β* in postischemic recovery of LVDP in WT and sEH null hearts. a, Hearts were administered vehicle, wortmannin (100 nmol/L), or LY294002 (5 μmol/L) before ischemia. Values represent mean ± SEM; n = 4 to 12; ‡$P<0.05$ vs vehicle control of same genotype; *$P<0.05$ vs WT. b, Representative immunoblots showing cardiac expression of phospho-GSK-3*β* and total GSK-3*β*. c, Representative immunoblots showing expression of phospho–GSK-3*β* and total GSK-3*β* at 5 minutes reperfusion after wortmannin (100 nmol/L) administration. d, Ratio of phospho–GSK-3*β* to total GSK-3*β* after 5 minutes reperfusion in the presence or absence of wortmannin. Values represent mean ± SEM; n = 4 per group; *$P<0.05$ vs WT.

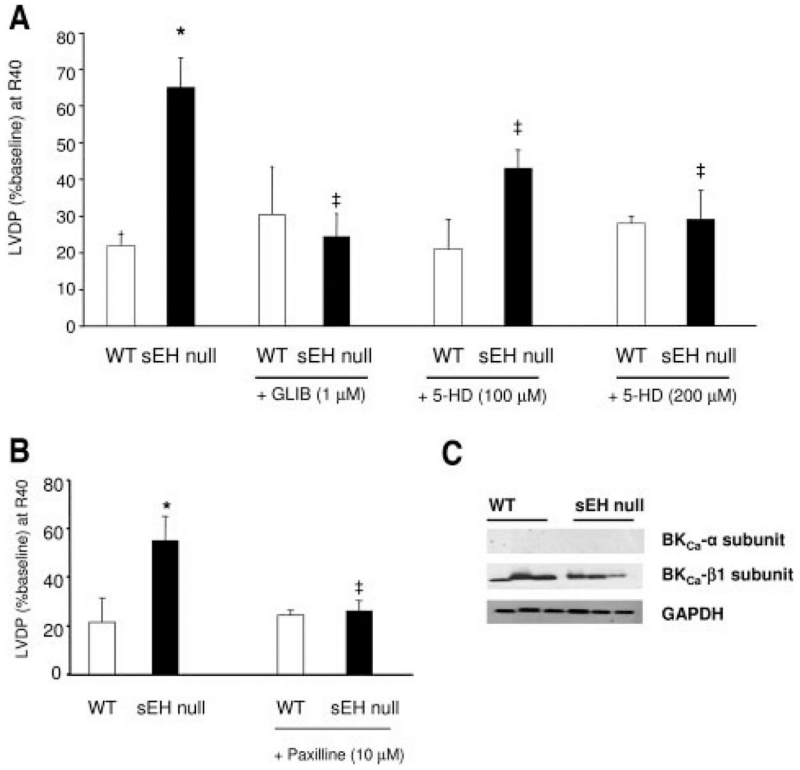

Figure 5.

Role of cardiac K^+ channels in postischemic recovery of LVDP in WT and sEH null hearts. a, Effects of K_{ATP} inhibitors on postischemic recovery of LVDP in WT and sEH null hearts. Hearts were administered vehicle, GLIB (1 μmol/L), or 5-HD (100, 200 μmol/L) before ischemia. b, Effects of BK_{Ca} inhibition on postischemic recovery of LVDP in WT and sEH null hearts. Hearts were administered vehicle or paxilline (10 μmol/L) before ischemia. Values represent mean ± SEM; n = 4 to 12 per group; *P<0.05 vs WT; ‡P<0.05 vs vehicle control of same genotype. c, Representative immunoblots showing expression of BK_{Ca}-α, BK_{Ca}-β1, and GAPDH in hearts from WT and sEH null mice.

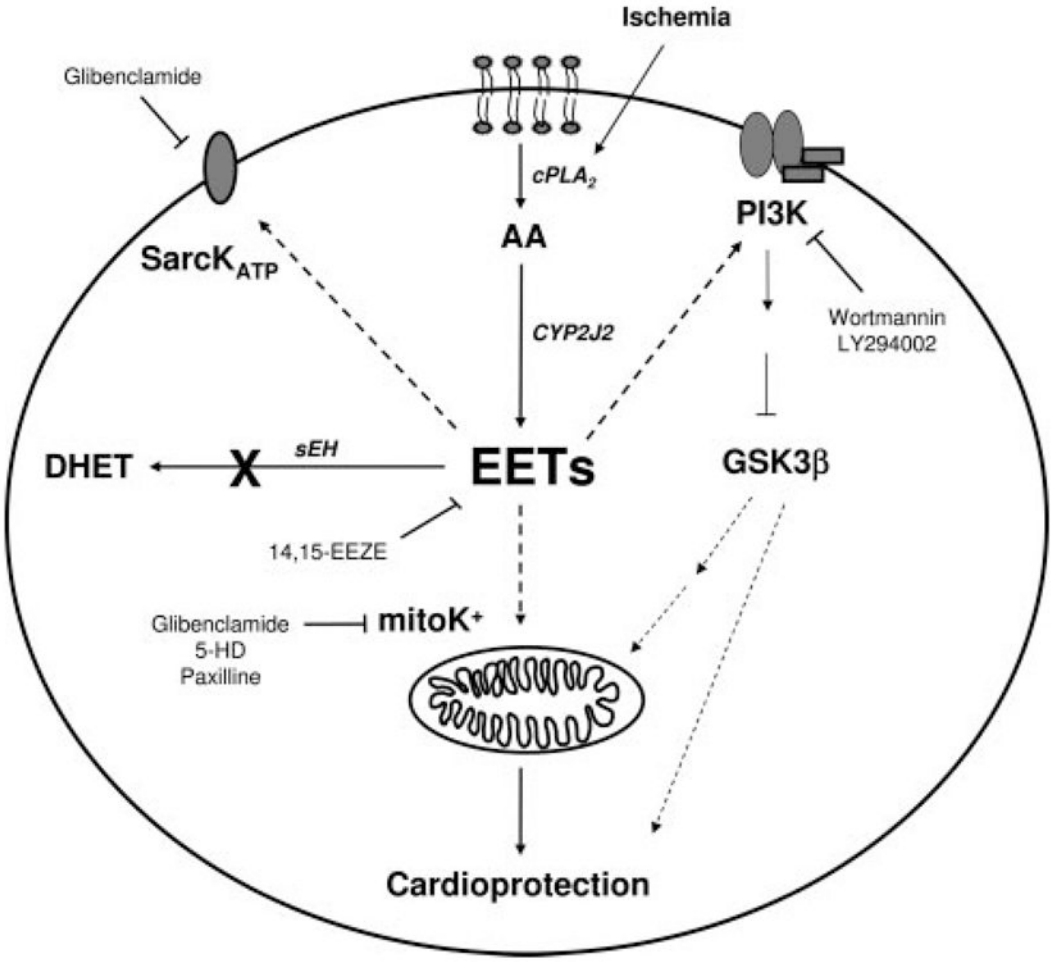

Figure 6.
Schematic of proposed mechanisms of cardioprotection in sEH null mice. EETs activate K^+ channels and the PI3K pathway leading to cardioprotection.

Table

Cardiac Parameters in sEH Null and WT Mice

Baseline	WT (n = 12 to 45)	sEH null (n = 12 to 45)
Body weight, g	28.3 ± 2.2	26.5 ± 1.4
Heart weight, mg	137.7 ± 9.5	128.4 ± 6.3
Heart/body wt, mg/g	5.3 ± 0.2	4.9 ± 0.2
Left ventricle free wall weight, mg	107.3 ± 7.5	97.5 ± 4.7
Right ventricle free wall weight, mg	24.3 ± 2.1	24.5 ± 2.0
Left atrium weight, mg	3.5 ± 0.4	3.5 ± 0.4
Right atrium weight, mg	2.6 ± 0.2	2.9 ± 0.3
M-mode echocardiogram		
Left ventricular end-diastolic dimension, mm	2.8 ± 0.1	3.0 ± 0.1
Left ventricular end-systolic dimension, mm	1.2 ± 0.2	1.4 ± 0.1
Fractional shortening, %	48.3 ± 0.4	52.6 ± 4.2
Septal wall thickness, mm	1.5 ± 0.5	1.4 ± 0.1
Posterior wall thickness, mm	1.6 ± 0.2	1.4 ± 0.1
HR, conscious, beats/min	669 ± 0.7	640 ± 57
Isolated perfused heart, preischemic		
LVDP, cmH_2O (Baseline)	108 ± 6	97 ± 5
Rate of contraction, dP/dt_{max}, cm H_2O/msec (Baseline)	6096 ± 497	5635 ± 364
Rate of relaxation, $-dP/dt_{min}$, cm H_2O/msec (Baseline)	−4461 ± 349	−3990 ± 270
HR, perfused, beats/min (Baseline)	366 ± 19	338 ± 9
Isolated perfused heart, postischemic		
LVDP, cmH_2O (R40)	23 ± 2	48 ± 4*
Rate of contraction, dP/dt_{max}, cm H_2O/msec (R40)	1511 ± 213	2942 ± 270*
Rate of relaxation, $-dP/dt_{min}$, cm H_2O/msec (R40)	−1034 ± 151	−1907 ± 164*
HR, perfused, beats/min (R40)	315 ± 27	317 ± 12

Body and heart weights were determined using an analytical balance; dimensions and percent fractional shortening were assessed by transthoracic echocardiography in conscious mice; hemodynamic parameters were measured in isolated-perfused hearts. Values are mean ± SEM,

*$P<0.05$ vs WT. HR indicates heart rate.