

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

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**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  $\mu$ mol/L), the ATP-sensitive K<sup>+</sup> channel (K<sub>ATP</sub>) inhibitor glibenclamide (1  $\mu$ mol/L), the mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>) inhibitor 5-hydroxydecanoate (100 to 200  $\mu$ mol/L), or the Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel (K<sub>Ca</sub>) inhibitor paxilline (10  $\mu$ mol/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-3 $\beta$  (GSK-3 $\beta$ ) 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<sup>+</sup> channels. (*Circ Res.* 2006;99:442-450.)

**Key Words:** arachidonic acid ■ cytochrome P450 ■ eicosanoid ■ ischemia/reperfusion

Arachidonic acid (AA), a polyunsaturated fatty acid normally found esterified to cell membrane glycerophospholipids, can be released by phospholipases in response to ischemia.<sup>1</sup> Free AA is then available for metabolism by prostaglandin H<sub>2</sub> synthases, lipoxygenases, and cytochrome P450 (CYP) monooxygenases to generate numerous metabolites, collectively termed eicosanoids.<sup>1</sup> 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.<sup>1</sup> The actions of EETs are terminated by conversion to the corresponding and less biologically active dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases.<sup>2</sup> Two major epoxide hydrolases are found in mammalian tissues, the microsomal epoxide hydrolase (mEH) and the soluble epoxide hydrolase (sEH or *Ephx2*).<sup>3</sup> Previous work has demonstrated that sEH is the main enzyme involved in the in vivo hydrolysis of EETs.<sup>4</sup>

EETs are important components of many intracellular signaling pathways in both cardiac and extracardiac tissues. For example, EETs activate large conductance Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels (BK<sub>Ca</sub>) in vascular smooth muscle cells resulting in hyperpolarization of the resting membrane potential and vasodilation of the coronary circulation.<sup>5</sup> This effect is diminished on hydrolysis of EETs to DHETs by sEH.<sup>6</sup> Other studies have shown that EETs display antiinflammatory, thrombolytic, and angiogenic properties within the vasculature.<sup>7-9</sup> 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.<sup>8-10</sup> In general, the effects of DHETs on these pathways are less pronounced.<sup>7,9</sup> Within the heart, EETs activate cardiac ATP-

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sensitive  $K^+$  ( $K_{ATP}$ ) channels,<sup>11,12</sup> enhance L-type calcium currents,<sup>13</sup> and improve postischemic recovery of left ventricular function.<sup>12,14,15</sup> 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.<sup>12</sup> The mechanism for the cardioprotection involved activation of mitochondrial  $K_{ATP}$  channels and the p42/p44-MAPK pathway.<sup>12</sup> 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.<sup>16</sup> 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).<sup>16</sup> 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.<sup>12,16</sup> Subcellular fractions were prepared from frozen mouse hearts. Immunoblots were probed with antibodies to sEH (generous gift from B.D.H.), GSK-3 $\beta$ , phospho-GSK-3 $\beta$ , p42/p44-MAPK, phospho-p42/p44-MAPK (Cell Signaling Technology, Inc, Beverly, Mass), BK $_{Ca}$ - $\alpha$ , BK $_{Ca}$ - $\beta$ 1, GAPDH, or actin C-11 (Santa Cruz Biotechnology, Santa Cruz, Calif).

### Fatty Acid Metabolism

Heart cytosolic fractions (2 mg protein/mL) were incubated with [<sup>14</sup>C]14,15-EET (100  $\mu$ mol/L) as described.<sup>17</sup> Products were extracted and analyzed by high-performance liquid chromatography.<sup>18</sup> 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.<sup>12,19</sup>

### Transthoracic Echocardiography, Assessment of Heart Anatomy

Two-dimensional guided M-mode echocardiography was performed using an HDI 5000 echocardiograph as described.<sup>20</sup> 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.<sup>12</sup> 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  $\mu$ mol/L), 11,12-EET (1  $\mu$ mol/L), 14,15-EET (1  $\mu$ mol/L), the sarcolemmal  $K_{ATP}$  (sarc $K_{ATP}$ ) and mitochondrial  $K_{ATP}$  (mito $K_{ATP}$ ) channel inhibitor glibenclamide (GLIB, 1  $\mu$ mol/L), the selective mito $K_{ATP}$  channel inhibitor 5-hydroxydecanoate (5-HD, 100 to 200  $\mu$ mol/L), the PI3K inhibitors wortmannin (200 nmol/L) or LY294002 (5  $\mu$ mol/L), the p42/p44-MAPK kinase (MEK) inhibitor PD98059 (10  $\mu$ mol/L), the BK $_{Ca}$  channel inhibitor paxilline (10  $\mu$ mol/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).

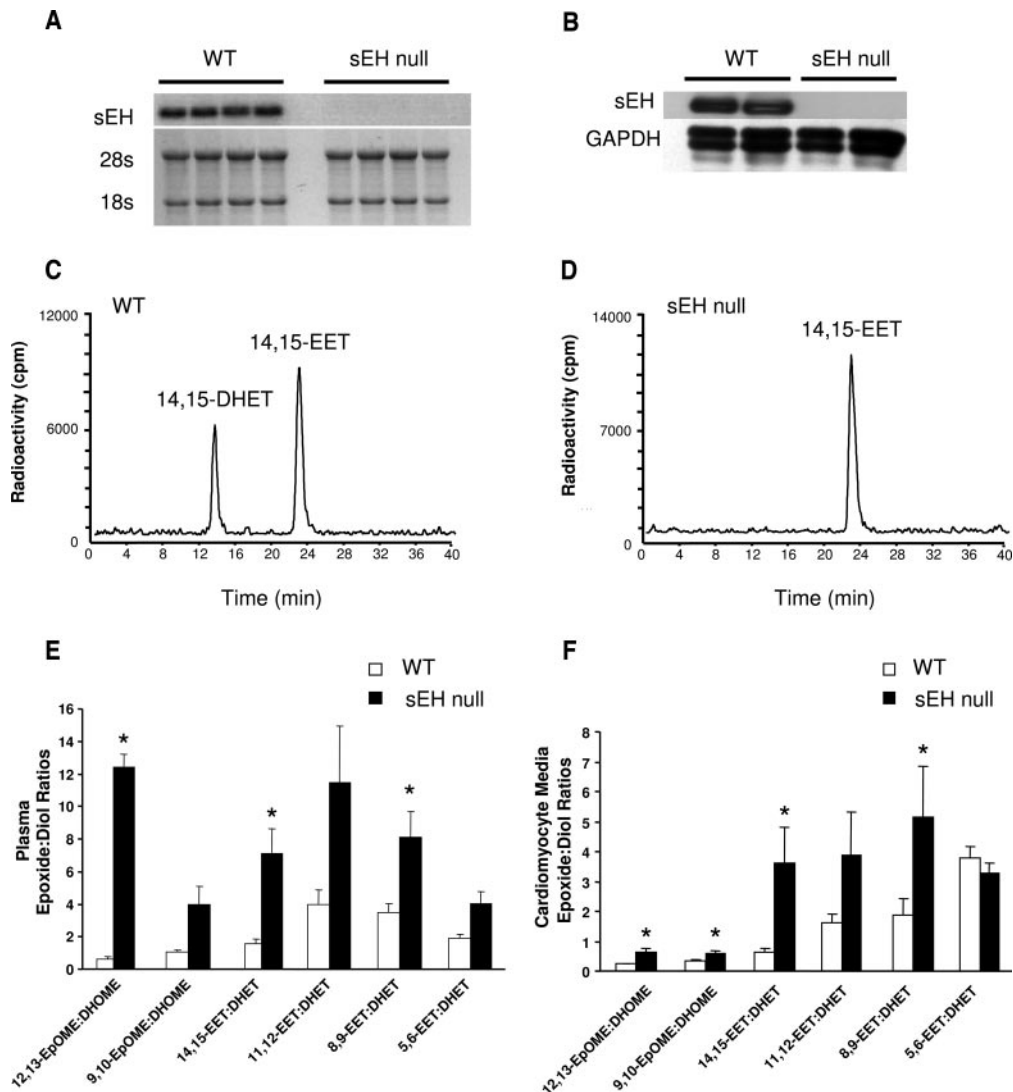
### Statistical Analysis

Values are expressed as mean $\pm$ 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:  $9.6 \pm 0.6$  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 dihydroxyoctadecenoic 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.



**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  $\pm$  SEM;  $n=4$  to 5 per group; \* $P<0.05$  vs WT.

### 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 ( $51 \pm 3\%$ ) than WT hearts ( $22 \pm 2\%$ ) (Figure 2b and 2c; Table). There were no differences between sEH null and WT hearts in time-to-onset of ischemic contracture ( $16.0 \pm 0.7$  minutes versus  $15.0 \pm 0.5$  minutes,  $P=0.15$ ) or maximal ischemic contracture ( $73 \pm 4$  cm H<sub>2</sub>O versus  $70 \pm 6$  cm H<sub>2</sub>O,  $P=0.55$ ). 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

**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 <sub>2</sub> O (Baseline)	108±6	97±5
Rate of contraction, dP/dt <sub>max</sub> , cm H <sub>2</sub> O/msec (Baseline)	6096±497	5635±364
Rate of relaxation, -dP/dt <sub>min</sub> , cm H <sub>2</sub> O/msec (Baseline)	-4461±349	-3990±270
HR, perfused, beats/min (Baseline)	366±19	338±9
Isolated perfused heart, postischemic		
LVDP, cmH <sub>2</sub> O (R40)	23±2	48±4*
Rate of contraction, dP/dt <sub>max</sub> , cm H <sub>2</sub> O/msec (R40)	1511±213	2942±270*
Rate of relaxation, -dP/dt <sub>min</sub> , cm H <sub>2</sub> O/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.

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

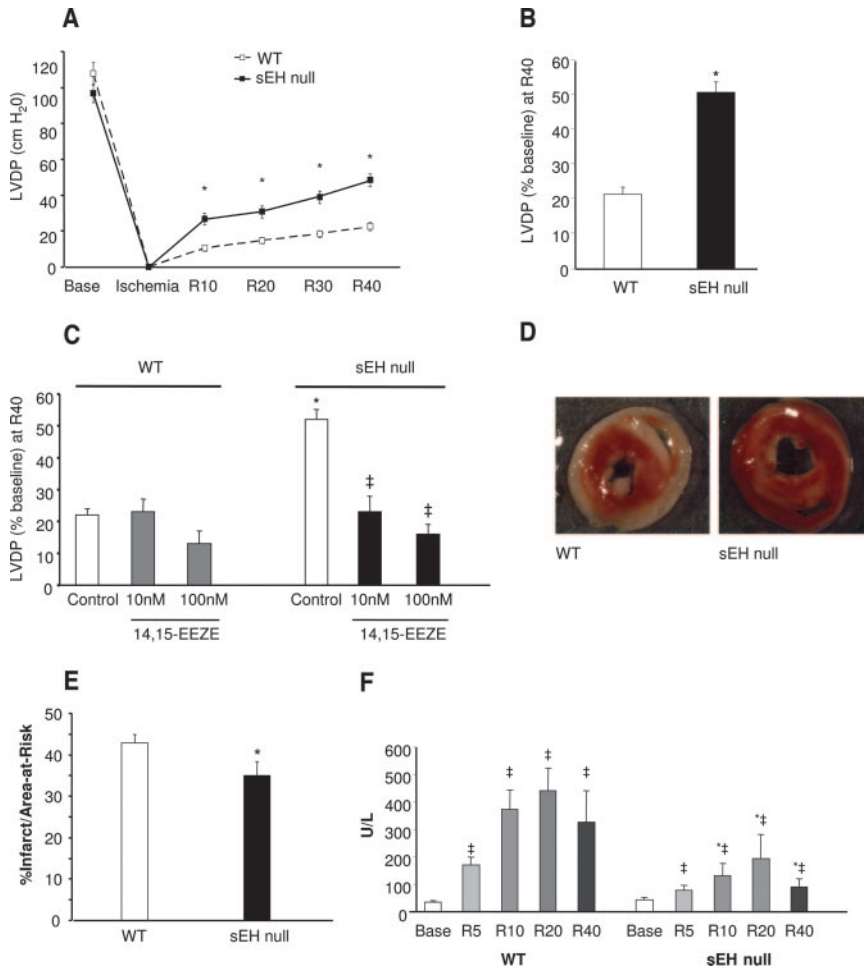
To determine whether the MAPK signaling pathway was involved in the cardioprotective mechanism, we examined the phosphorylation status of p42/p44-MAPK in WT and sEH

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





**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  $\pm$  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  $\pm$  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  $\pm$  SEM; n=5; \* $P$ <0.05 vs WT. f, LDH activity in perfusates from WT and sEH null hearts. Values represent mean  $\pm$  SEM; n=6 to 12 per group; ‡ $P$ <0.05 vs baseline of same genotype; \* $P$ <0.05 vs WT at same time point.

and sEH null hearts at baseline, during ischemia, and during reperfusion. There were no significant differences in the expression of phospho-GSK-3 $\beta$  between WT and sEH null hearts under basal conditions (Figure 4b). Interestingly, levels of phospho-GSK-3 $\beta$  were significantly higher in sEH null hearts compared with WT hearts at 5 minutes of reperfusion (Figure 4b). Likewise, the ratio of phospho-GSK-3 $\beta$  to total GSK-3 $\beta$  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-3 $\beta$  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-3 $\beta$  activation in cardioprotection,<sup>21</sup> these data suggest one possible mechanism for the observed postischemic recovery in sEH null mice.

### Role of K<sup>+</sup> Channels in Postischemic Functional Recovery

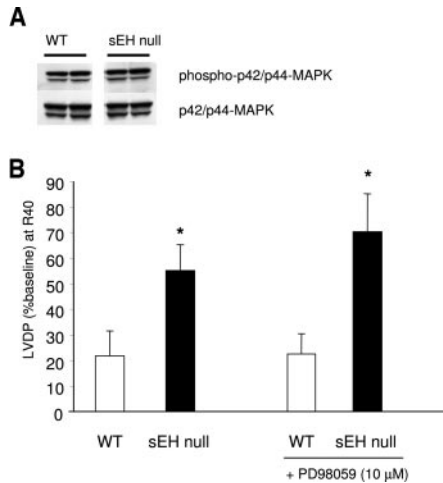
To determine whether K<sub>ATP</sub> channels were involved in the cardioprotective mechanism, we conducted experiments in the presence or absence of the sarcK<sub>ATP</sub> and mitoK<sub>ATP</sub> inhibitor GLIB or the selective mitoK<sub>ATP</sub> inhibitor 5-HD. Neither GLIB nor 5-HD had significant 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<sub>Ca</sub> channels in the cardioprotective phenotype, we examined the effects of the specific BK<sub>Ca</sub> inhibitor paxilline (100  $\mu$ mol/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<sub>Ca</sub>- $\beta$ 1 subunit between WT and sEH null hearts (Figure 5c). Expression of the BK<sub>Ca</sub>- $\alpha$  subunit was low-undetectable in both WT and sEH null hearts, consistent with a recent publication.<sup>22</sup>

### Discussion

Cardiac CYP epoxygenases convert AA to biologically active EETs that have well established effects within the cardiovascular system.<sup>12,18</sup> The activity of these compounds is terminated mainly through conversion to less active DHETs by sEH.<sup>2</sup> Recent studies demonstrate that CYP epoxygenase-derived eicosanoids can affect cardiomyocyte function<sup>11,13,14</sup> and improve postischemic LVDP recovery.<sup>12,14,15</sup> In addition, EET biosynthesis is enhanced in stenosed coronary arteries<sup>23</sup> and during cardiac ischemia/reperfusion injury.<sup>24</sup> The data

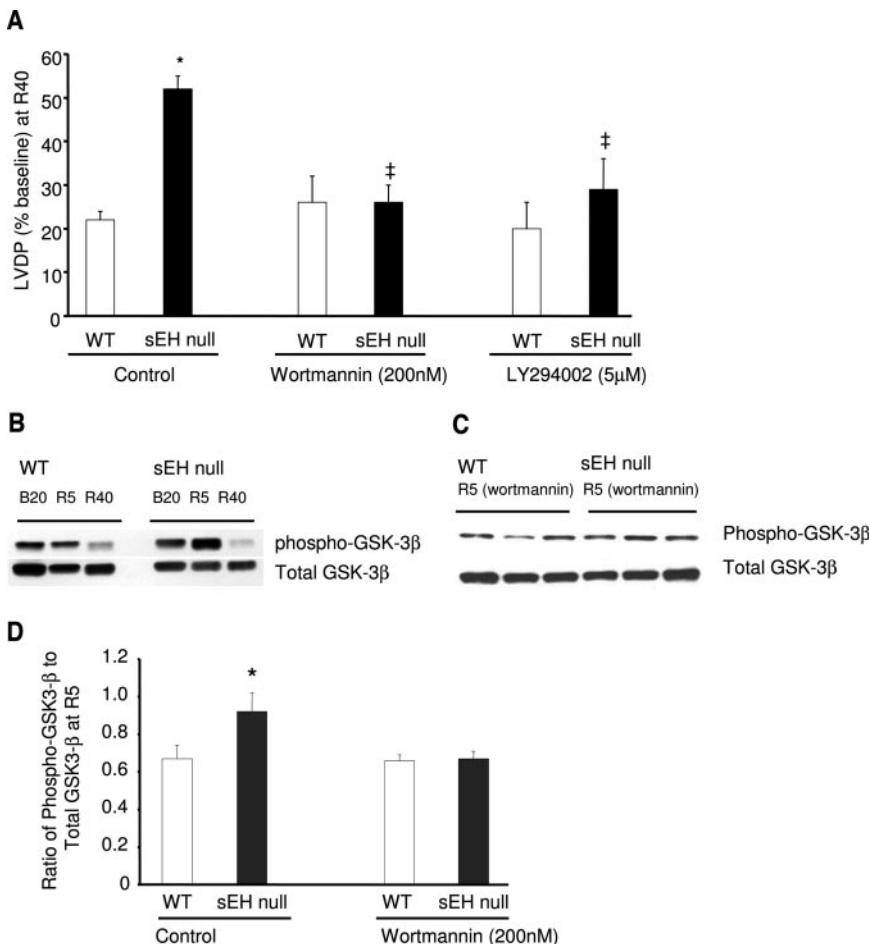


**Figure 3.** Activation status of p42/p44-MAPK in WT and sEH null hearts. a, Representative immunoblots showing expression of phospho-p42/p44-MAPK and total p42/p44-MAPK in hearts after 5 minutes reperfusion. Similar results were obtained at other time points. b, Effects of MEK inhibition on postischemic 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.

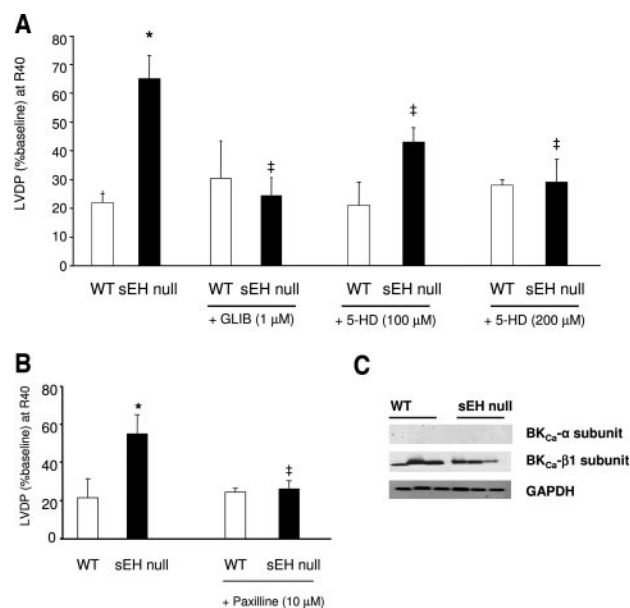
presented herein provide further evidence that EETs are involved in the cardioprotective response. We demonstrate, for the first time, that targeted disruption of the *Ephx2* gene leads to improved recovery of left ventricular function and

reduced infarction after ischemia. Moreover, the putative EET receptor antagonist 14,15-EEZE abolishes the cardioprotection 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 glycerophospholipids where they are generally considered inactive until their release.<sup>25</sup> 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<sub>2</sub>, leading to the release of bioactive eicosanoids from glycerophospholipids.<sup>26</sup> The results in the current article are consistent with our recent findings that mice overexpressing human CYP2J2 in the heart



**Figure 4.** Role of PI3K and GSK-3β 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-3β and total GSK-3β. c, Representative immunoblots showing expression of phospho-GSK-3β and total GSK-3β at 5 minutes reperfusion after wortmannin (100 nmol/L) administration. d, Ratio of phospho-GSK-3β to total GSK-3β 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  $\mu$ M), or 5-HD (100, 200  $\mu$ M) before ischemia. **b**, Effects of BK<sub>Ca</sub> inhibition on postischemic recovery of LVDP in WT and sEH null hearts. Hearts were administered vehicle or paxilline (10  $\mu$ M) before ischemia. Values represent mean  $\pm$  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<sub>Ca</sub>- $\alpha$ , BK<sub>Ca</sub>- $\beta$ 1, and GAPDH in hearts from WT and sEH null mice.

have normal heart anatomy and function at baseline, but have elevated cardiomyocyte EET levels and improved postischemic functional recovery.<sup>12</sup>

AA can be metabolized by CYP epoxygenases and CYP  $\omega$ -hydroxylases to products that have different physiologic effects. For example, EETs have potent vasodilatory properties and 20-HETE has vasoconstrictive effects.<sup>1</sup> 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  $\omega$ -hydroxylases results in reduction of infarction after ischemic injury, suggesting that 20-HETE has detrimental effects within the heart.<sup>27,28</sup> 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<sup>29</sup> and altered cardiac electrical activity.<sup>30</sup> 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.<sup>31</sup> 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,<sup>32</sup> 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.<sup>12,14</sup>

Hydrolysis of EETs by sEH is dependent on both regio- and stereoselective parameters.<sup>33</sup> 14,15-EET is the preferred sEH substrate, and 5,6-EET is an extremely poor substrate for this enzyme.<sup>33</sup> 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.<sup>12</sup> 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.<sup>34</sup> The PI3K signaling pathway has been shown to be involved in cardioprotective responses after ischemia/reperfusion.<sup>35,36</sup> Signaling through PI3K promotes a prosurvival mechanism involving recruitment of downstream kinases such as GSK-3 $\beta$ .<sup>34,35</sup> 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-3 $\beta$  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.<sup>8</sup> Recent evidence suggests that convergence of multiple cardioprotective signaling pathways onto GSK-3 $\beta$  may represent an integrated mechanism of cellular protection.<sup>21</sup> It was proposed that this convergence targets the mitochondrial permeability transition pore (MPTP) complex limiting its opening.<sup>21</sup> Inhibition of MPTP induction early during reperfusion has cardioprotective effects.<sup>35</sup>

A large body of research suggests a role for  $K_{ATP}$  channels in cardioprotection.<sup>37</sup> Cardiomyocyte studies have demonstrated that EETs enhance both sarco $K_{ATP}$  and mito $K_{ATP}$  channel activities.<sup>11,12</sup> In the current study, administration of either



the sarcK<sub>ATP</sub> channel inhibitor glibenclamide or the mitoK<sub>ATP</sub> channel inhibitor 5-HD attenuated the improved postischemic recovery of sEH null hearts. The precise identity of the mitoK<sub>ATP</sub> 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<sub>Ca</sub> channels in protection against ischemia/reperfusion injury.<sup>38</sup> EETs are known activators and  $\omega$ -HETEs are known inhibitors of these channels in vascular smooth muscle cells.<sup>1,5</sup> Newly identified K<sub>Ca</sub> channels in cardiac mitochondria (mitoK<sub>Ca</sub>) are thought to work in concert with mitoK<sub>ATP</sub> and other mitochondrial proteins to mediate cardioprotection.<sup>38,39</sup> Our experiments demonstrate that the BK<sub>Ca</sub> 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<sup>+</sup> uptake and, in turn, reducing Ca<sup>2+</sup> overload, with a net effect of protecting mitochondrial function.<sup>38,39</sup>

We postulate that the enhanced functional recovery observed in the sEH null mice entails modulation of both PI3K and mitochondrial K<sup>+</sup> 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.<sup>40–42</sup> 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.

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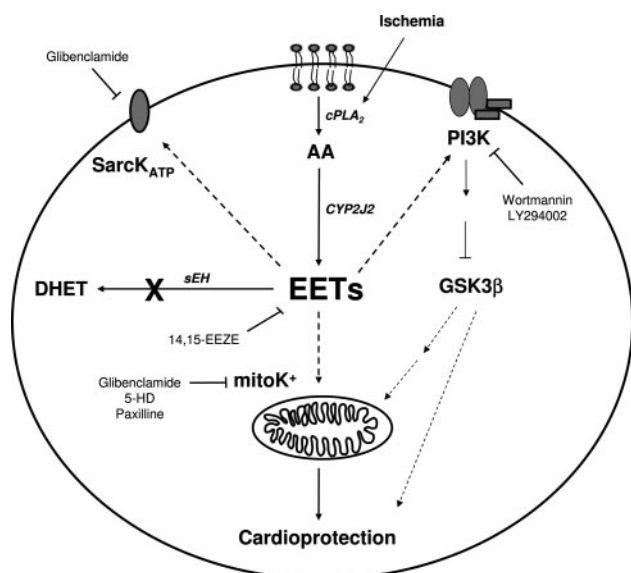
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## Disclosures

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**Figure 6.** Schematic of proposed mechanisms of cardioprotection in sEH null mice. EETs activate K<sup>+</sup> channels and the PI3K pathway leading to cardioprotection.



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

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## EXPANDED MATERIALS AND METHODS

### Animals

Mice with the targeted disruption in of the *Ephx2* gene were obtained from Chris Sinal, Dalhousie University, Halifax, NS, Canada.<sup>1</sup> These mice, backcrossed onto a C57BL6 genetic background for five generations, were utilized to re-derive a new colony at NIEHS by embryo transfer (Charles River Laboratories). For breeding, *Ephx2*<sup>+/+</sup> (wild type, WT) and *Ephx2*<sup>-/-</sup> (sEH null) mice were obtained by crossing male and female *Ephx2*<sup>+/-</sup> (heterozygous) animals. Routine genotyping of sEH null mice was performed according to the method of Sinal et al.<sup>1</sup> The following primers were utilized: F1, 5'-tggcagcaccctaactcttaggttc-3'; R2, 5'-tgcacgctggcattttaacaccag-3'; and R3, 5'-ccaatgacaagacgctggcg-3'. Primers F1/R2 predict a 338-base pair product for the WT allele. For the null allele, primers F1/R3 predict a 295-base pair product, which is preferentially amplified over the predicted 1477-base pair product for F1/R2. All studies used male and female mice aged 4-6 months, weighing 25-35g. All experiments were approved by the NIEHS Animal Care and Use Committee.

### Northern Analysis, Immunoblotting

Hearts were dissected, quickly rinsed in ice cold sterile PBS and snap frozen in liquid nitrogen. Total RNA was isolated using 1.5 ml of TRIzol reagent (Invitrogen Burlington, ON) as per the manufacturer's instructions. 100 µg of total RNA from each sample was further purified using RNeasy mini extraction columns (Qiagen, Mississauga, ON). Northern blot analysis was performed as described, with minor modifications.<sup>2</sup> Briefly, purified total RNA (5 µg) was separated by electrophoresis in a denaturing 1.1% agarose/0.22 M formaldehyde gel and transferred to Immobilon-Ny<sup>+</sup> nylon membranes



(Millipore, Billerica, MA) in 20X SSC buffer. After crosslinking with UV light and drying for 1 hr at 65 °C, the blots were hybridized with the full length murine sEH cDNA probe (a generous gift from Frank Gonzalez NCI/NIH, Bethesda, MD) labeled with [ $\alpha$ - $^{32}$ P]dCTP by the random primer method. After hybridization overnight at 65°C in PerfectHyb buffer (Sigma-Aldrich), the blots were washed once with 2X SSC/0.5% SDS for 15 min at 65°C, followed by two identical washes for 30 min each. Blots were exposed to storage phosphor screens, images were detected using a Storm 840 PhosphorImager (Amersham Biosciences, Baie d'Urfé, QC) and results quantitated using ImageQuant 5.2 software (Amersham Biosciences). sEH expression was normalized to 28s rRNA as detected by ethidium bromide staining. Subcellular fractions were prepared from frozen mouse hearts and immunoblotting was performed as described.<sup>1,3,4</sup> Briefly, cytosolic protein (50 $\mu$ g) from individual hearts was resolved on 12% sodium dodecylsulfate-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with antibodies to either sEH (1:500) (a gift from Bruce Hammock, University of California, Davis, CA) or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

### **Fatty Acid Metabolism**

Heart cytosolic fractions (2mg protein/ml) were incubated with [1- $^{14}$ C]14,15-EET (100 $\mu$ M) as described.<sup>5</sup> Products were extracted and analyzed by high-performance liquid chromatography.<sup>3,6</sup> Epoxygenase activity was calculated as the rate of 14,15-DHET produced/mg protein/min at 37°C.

Cardiomyocyte cell culture media, adult plasma and heart perfusate buffer were analyzed for epoxy and dihydroxy fatty acid derivatives of AA and linoleic acid (LA)

using established HPLC/MS/MS methods.<sup>7</sup> Cardiomyocytes were isolated from neonatal sEH null and WT hearts as described<sup>8</sup> and cultured on 1% gelatin-coated plates at 37°C in Dulbecco's modified Eagle's medium (DMEM/F12) containing 10% fetal bovine serum under an atmosphere of 5% CO<sub>2</sub>/95% air for 24 hours. Media samples were spiked with 5mg butylated hydroxytoluene and the following analytical surrogates in methanol: 10,11-dihydroxyheptadecanoic acid (10,11-DHHep), 10,11-dihydroxynonadecanoic acid (10,11-DHN), 10,11-epoxyheptadecanoic acid (10,11-EpHep), octadeuterated-6-keto-prostaglandin F<sub>1</sub> (d8-6-keto-PGF<sub>1α</sub>), each at a final concentration of 800nM. Media samples were vortexed 1min with 0.5ml of ethyl acetate and centrifuged. The organic phase was isolated and the extraction was repeated. The combined organic phases were reduced to dryness under nitrogen. The residue was dissolved in 50μl of methanol containing 1-cyclohexyl-3-docosahexanoic acid urea at 800nM as an internal standard. Plasma samples were extracted by a solid phase extraction method. First, 60mg Oasis-HLB SPE cartridges (Waters Milford, MA) were preconditioned with 2mL of 2.5mM phosphoric acid-10% methanol (pH 3.8). Samples were spiked with surrogates as described above and immediately diluted 1:1 v/v with 2.5mM phosphoric acid. After sample loading, cartridges were washed with 2mL of 2.5mM phosphoric acid-10% methanol (pH 3.8) and analytes were eluted in 2mL of ethyl acetate.

Oxylipids in 20μL extract aliquots were separated by reverse phase HPLC on a 2.1 x 150mm, 5μM Luna C18(2) column (Phenomenex, Torrance, CA) and quantified using a Quattro Ultima tandem quadrupole mass spectrometer (Micromass, Manchester, UK) with negative mode electrospray ionization and multiple reaction

monitoring as previously described.<sup>9</sup> The relative response ratios of analytes were used to calculate analyte concentrations while correcting for surrogate losses. Surrogate recoveries were evaluated by quantification against the internal standards. Epoxides were corrected for 10,11-EpHep, while diols and 20-hydroxyeicosatrienoic acid (20-HETE) were corrected for 10,11-DHN recoveries. Spontaneous hydrolysis of 10,11-EpHep was <3% for all assays. Surrogate recoveries were >70% for all reported results. The analysis of reagent blanks, matrix spikes, and analytical replicates were used to document method stability during this study.

### **Transthoracic Echocardiography, Assessment of Heart Anatomy**

Two-dimensional guided M-mode echocardiography was performed using an HDI 5000 echocardiograph as described.<sup>10</sup> sEH null mice and WT littermate controls were then sacrificed, and hearts removed, dissected, and weighed.

### **Isolated-Perfused Hearts**

Hearts were perfused in the Langendorff mode as described.<sup>4</sup> Briefly, hearts from sEH null and age/sex-matched WT littermate controls were cannulated and perfused in a retrograde fashion at constant pressure (100cmH<sub>2</sub>O) with continuously aerated (95%O<sub>2</sub>/5%CO<sub>2</sub>) Krebs-Henseleit buffer at 37°C. For assessment of cardiac function, a balloon-tipped catheter inserted into the left ventricle through the left atrium was connected to a pressure transducer. The intraventricular balloon pressure and volume were adjusted to give an initial end-diastolic pressure of 10cmH<sub>2</sub>O. A PowerLab system (AD Instruments) was used to process data. Hearts were perfused with buffer for a 40min stabilization period, then subjected to 20min global no-flow ischemia, followed by 40min reperfusion. For some experiments, hearts were stabilized for

20min, then perfused with either the putative EET receptor antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE, 10 or 100nM), 8,9-,11,12-, 14,15-EET (1 $\mu$ M), the sarcolemmal  $K_{ATP}$  (sarc $K_{ATP}$ ) and mitochondrial  $K_{ATP}$  (mito $K_{ATP}$ ) channel inhibitor glibenclamide (GLIB, 1 $\mu$ M), the selective mito $K_{ATP}$  channel inhibitor 5-hydroxydecanoate (5-HD, 100 or 200 $\mu$ M), the PI3K inhibitor wortmannin (200 nM), the PI3K inhibitor LY294002 (5  $\mu$ M), the MEK1/2 inhibitor PD98059 (10 $\mu$ M), the BK $_{Ca}$  channel inhibitor paxilline (10 $\mu$ M) or vehicle for 20min, then subjected to 20min ischemia and 40min 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 amount of infarction, after 2h reperfusion, hearts were incubated with a 1% solution of 2,3,5-triphenyltetrazolium chloride dissolved in Krebs-Henseleit buffer at 37°C for 10min. The hearts were fixed in formalin and then were cut into thin cross-sectional slices. The area of necrosis was quantitated by measuring the stained area (red, live tissue) versus the unstained area (white, necrotic). LDH activity was assessed in heart perfusate buffer using a commercially available assay (Roche Molecular Biochemicals, USA).

### **Protein Expression and Activation of MAPK, GSK-3 $\beta$ and BK $_{Ca}$ Subunits**

The expression of total glycogen synthase kinase 3-beta (GSK-3 $\beta$ ) and phospho-GSK-3 $\beta$  was determined in hearts at different times during the ischemia-reperfusion protocol. Individual hearts from either sEH null or WT mice were snap frozen in liquid nitrogen after 20min of perfusion (B20), or after 5min (R5) or 40min of postischemic reperfusion (R40). Protein (50 $\mu$ g) from the 10,000g supernatant of individual hearts was resolved on 12% sodium dodecylsulfate-polyacrylamide gels, transferred to



nitrocellulose membranes and immunoblotted with antibodies to either GSK-3 $\beta$  (1:400), phospho-GSK-3 $\beta$  (1:400) (Cell Signaling Technology, Inc., Beverly, MA) or  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA). Relative band intensities, expressed in arbitrary units of phospho-GSK-3 $\beta$  to total GSK-3 $\beta$ , were assessed by densitometry using a Chemilmager 4000 Imaging System (Alpha Innotech Corp., San Leandro, CA).<sup>17, 20</sup> Protein expression was determined after 20min of perfusion (B20), or after 5min (R5) or 40min of postischemic reperfusion (R40). Immunoblots from individual hearts were assessed with antibodies to GSK-3 $\beta$  (1:400), phospho-GSK-3 $\beta$  (1:400) (Cell Signaling Technology, Inc., Beverly, MA), p42/p44 MAPK (1:1000), phospho-p42/p44 MAPK (1:1000) (Cell Signaling Technology, Inc., Beverly, MA), BK<sub>Ca</sub>- $\alpha$ , BK<sub>Ca</sub>- $\beta$ 1 or actin C-11 (Santa Cruz Biotechnology, Santa Cruz, CA). Relative band intensities, expressed in arbitrary units of phospho-protein to total protein, were assessed by densitometry using a Chemilmager 4000 System (Alpha Innotech Corp., San Leandro, CA).

### Statistical Analysis

Values are expressed as mean  $\pm$  standard error of the mean (SEM). Data were analyzed by ANOVA or Student's t-test using SYSTAT software (SYSTAT Inc.). Values were considered significantly different if  $P < 0.05$ .

**Supplemental Table 1: Oxylipid Concentrations in Plasma From WT and sEH null mice**

	Concentration (nM)	
	WT	sEH null
12,13-EpOME	16.3 ± 1.2	109 ± 19 **
9,10-EpOME	12 ± 0.52	39.3 ± 12 *
12,13-DHOME	27.5 ± 3.7	8.8 ± 1.6 **
9,10-DHOME	11.9 ± 1.4	10.4 ± 2.7
14,15-EET	1.19 ± 0.14	3.8 ± 1.4
11,12-EET	1.82 ± 0.4	4.48 ± 1.8
8,9-EET	2.23 ± 0.41	3.54 ± 1
5,6-EET	1.53 ± 0.17	2.87 ± 0.94
14,15-DHET	0.805 ± 0.15	0.472 ± 0.09 *
11,12-DHET	0.485 ± 0.1	0.344 ± 0.06
8,9-DHET	0.655 ± 0.1	0.408 ± 0.05 *
5,6-DHET	0.825 ± 0.08	0.656 ± 0.12

Results are means ± SEM; differences between WT and sEH null mice are indicated (\* p < 0.1, \*\* p < 0.05)

**Supplemental Table 2: Oxylipid Concentrations in Cardiomyocyte Media From WT and sEH null Mice**

	Concentration (nM)	
	WT	sEH null
12,13-EpOME	0.484 ± 0.08	1.26 ± 0.23 **
9,10-EpOME	0.608 ± 0.07	1.1 ± 0.17 **
12,13-DHOME	1.94 ± 0.12	2.09 ± 0.17
9,10-DHOME	1.70 ± 0.1	1.89 ± 0.14 *
14,15-EET	0.309 ± 0.03	0.929 ± 0.31
11,12-EET	0.809 ± 0.12	1.61 ± 0.62
8,9-EET	0.985 ± 0.22	1.81 ± 0.66
5,6-EET	1.64 ± 0.23	2.22 ± 0.33
14,15-DHET	0.495 ± 0.05	0.309 ± 0.05 **
11,12-DHET	0.526 ± 0.03	0.445 ± 0.05
8,9-DHET	0.488 ± 0.05	0.392 ± 0.05 *
5,6-DHET	0.473 ± 0.04	0.688 ± 0.11 **

Results are means ± SEM; differences between WT and sEH null mice are indicated (\* p < 0.1, \*\* p < 0.05)

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