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.[1] 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, $Ephx2^{+/+}$ (wild type, WT) and $Ephx2^{-/-}$ (sEH null) mice were obtained by crossing male and female $Ephx2^{+/-}$ (heterozygous) animals. Routine genotyping of sEH null mice was performed according to the method of Sinal et al.[1] The following primers were utilized: F1, 5'-tggcacgaccctaatcttaggttc-3'; R2, 5'-tgcacgctggcattttaacaccag-3'; and R3, 5'-ccaatgacaagacgctgggcg-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.[2] 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^+ 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 [α-^{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.[1,3,4] Briefly, cytosolic protein (50µg) 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 (100µM) as described.[5] Products were extracted and analyzed by high-performance liquid chromatography.[3,6] 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.[7] Cardiomyocytes were isolated from neonatal sEH null and WT hearts as described[8] 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_2/95% 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 F1 (d8-6-keto-$PGF_{1\alpha}$), 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.[9] 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.[10] 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.[4] Briefly, hearts from sEH null and age/sex-matched WT littermate controls were cannulated and perfused in a retrograde fashion at constant pressure (100cmH_2O) with continuously aerated (95%O_2/5%CO_2) 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 10cm$H_2$0. 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 (1µM), the sarcolemmal K_{ATP} (sarcK_{ATP}) and mitochondrial K_{ATP} (mitoK_{ATP}) channel inhibitor glibenclamide (GLIB, 1µM), the selective mitoK_{ATP} channel inhibitor 5-hydroxydecanoate (5-HD, 100 or 200µM), the PI3K inhibitor wortmannin (200 nM), the PI3K inhibitor LY294002 (5 µM), the MEK1/2 inhibitor PD98059 (10µM), the BK_{Ca} channel inhibitor paxilline (10µM) 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-3β and BK_{Ca} Subunits

The expression of total glycogen synthase kinase 3-beta (GSK-3β) and phospho-GSK-3β 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 (50µg) 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-3β (1:400), phospho-GSK-3β (1:400) (Cell Signaling Technology, Inc., Beverly, MA) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Relative band intensities, expressed in arbitrary units of phospho-GSK-3β to total GSK-3β, were assessed by densitometry using a ChemiImager 4000 Imaging System (Alpha Innotech Corp., San Leandro, CA). [17, 20] 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-3β (1:400), phospho-GSK-3β (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_{Ca}-α, BK_{Ca}-β1 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 ChemiImager 4000 System (Alpha Innotech Corp., San Leandro, CA).

Statistical Analysis

Values are expressed as mean ± 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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