Soluble Epoxide Hydrolase Gene Deletion is Protective Against Myocardial Ischemia Reperfusion Injury In Vivo

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ABSTRACT # 479.3

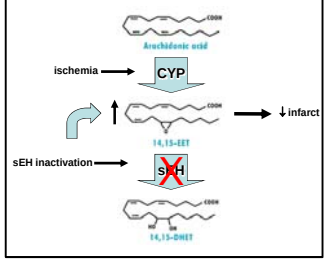
Epoxyeicosatrienoic acids (EETs) are metabolized by soluble epoxide hydrolase (sEH). Targeted disruption of the sEH gene enhances post-ischemic contractile function in isolated hearts and exogenous administration of EETs limits myocardial infarct size in vivo. We tested the hypothesis that sEH gene deletion is cardioprotective.

Male C57BL\6J wild-type (WT) mice or sEH knockout (sEHKO) mice were subjected to 40 min coronary artery occlusion and 120 min reperfusion. Area-at-risk (AAR) and infarct size (I) were assessed using fluorescent microspheres and triphenyl tetrazolium chloride. Infarct size is expressed as I/AAR (mean SEM). Myocardial sEH protein content was assessed by immunoblot and its distribution by immunohistochemistry (IHC).

I/AAR was reduced by 25 9% in sEHKO compared to WT (37 3% n=10 vs. 49 3% n=8, p<0.05). AAR/heart volume was comparable between groups (27 4% vs. 29 4%). Western blot confirmed the absence of sEH in sEHKO and its presence in WT hearts. IHC was positive for sEH in cardiomyocytes in WT but not sEHKO mice.

Cardioprotection from targeted deletion of sEH is likely due to increased endogenous myocardial EET levels. Infusion of EETs or inhibition of sEH may serve as a novel therapeutic option for cardioprotection during coronary revascularization and cardiac transplantation. Support: VA Merit Review 317 (DMVW) and RO1 NS44313 (NJA).

CONCEPT



BACKGROUND

Arachidonic acid (AA) is released from membrane phospholipids in response to a variety of pathophysiologic and pharmacologic stimuli, including myocardial ischemia. Free AA is metabolized by three pathways: cyclooxygenase, lipooxygenase and cytochrome P450 monooxygenase (CYP). CYP metabolizes AA to 4 biologically active eicosanoids (epoxyeicosatrienoic acids EET): 5,6-EET; 8,9-EET, 11,12-EET and 14,15-EET. EETs have anti-inflammatory and anti-thrombotic effects, are coronary vasodilators, and reduce myocardial ischemic injury (1-4). The biological activity of EETs is terminated by hydration into less active dihydroeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). Functional recovery is improved in isolated hearts from sEHKO mice after global ischemia (5).

HYPOTHESIS

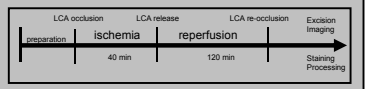
Augmentation of endogenous EETs by inactivation of sEH reduces infarct size/AAR in vivo.

METHOD

Animals: Male C57BL\6J or sEHKO mice with targeted deletion of the sEH encoding gene *EPHX2* received treatment in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, National Research Council; National Academy Press, 1996) and with IACUC approval.

Regional Myocardial Ischemia-Reperfusion Injury.

Mice were anesthetized with isoflurane and intubated with a 20G plastic IV catheter and ventilated. ECG and rectal temperature were monitored. The animals were positioned in a right lateral decubital position and rectal temperature was maintained at 37ºC. A PE-10 catheter was inserted into the jugular vein for drug infusion. A left-sided thoracotomy was performed in the 4th intercostal space. A ligature was placed around the Left Coronary Artery (LCA). LCA-occlusion was confirmed with persistent ECG changes during occlusion and visual paling of the left ventricle (LV). Reperfusion was confirmed with visual hyperemia of the LV and return of the ECG to baseline. After LCA re-occlusion, fluorescent microspheres were infused via needle puncture of the LV apex and the heart was excised. The ventricles were sliced into seven sections (d=1 mm) for imaging and staining. The microspheres delineate the non-perfused area (AAR). The infarct size (I) was determined by staining in 1% 2,3,5 triphenyl tetrazolium chloride (TTC) followed by 10% formalin bath overnight .



Immunohistochemistry.

Samples (LV) were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5-µm sections and mounted on superfrost glass slides. Sections were kept at 60°C overnight and then de-paraffinized with xylene followed by washing in 100%, 96%, 80%, and 70% ethanol. Heat unmasking of epitopes was done by boiling the samples three times in citrate buffer (pH-6) (600 W microwave). After cooling down to room temperature and 1 h of blocking in 5% dry milk, slides were incubated overnight at room temperature with primary antibodies (rabbit anti-sEH antibody, 1:50, Santa Cruz; sheep anti-TH antibody, 1:100, Pel-Freez). After washing in PBS, sections were incubated with secondary FITC-conjugated goat anti-rabbit IgG (1:100 in 5% dry milk, Invitrogen) and Alexa Fluo 597 donkey anti-sheep IgG (1:200 in 5% dry milk, Invitrogen) for 4 h. After washing, sections were placed in an autofluorescence-reduction solution (10 mM $CuSO_4$, 50 mM ammonium acetate) for 30 min, and then mounted with Slow Fade Gold Antifade Reagent with DAPI (Invitrogen). The sections were viewed on Zeiss Axiovert 200 fluorescent microscope. Negative controls included sections in which the primary antibodies were omitted, incubated with secondary antibodies only, or sections in which both primary and secondary antibodies were omitted. Further negative controls included use of sEH primary antibody after preincubation with its specific blocking peptide.

Western Blot.

Heart or cardiomyocyte proteins in SDS sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 0.15 M β-mercaptoethanol, 0.02% bromphenol blue) were separated on 4-20% linear gradient SDS–polyacrylamide gels (Bio-Rad) in a minigel apparatus (Mini-PROTEAN 3, Bio-Rad) and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 7.5,150 mM NaCl, 0.05% Tween 20) for 60 minutes at room temperature and incubated overnight at 4°C with primary rabbit anti-sEH antibody (1:2000 in 5% dry milk, Santa Cruz). The antigens were detected by the luminescence method (ECL-plus Western blotting detection kit, Amersham) with peroxidase-linked anti-rabbit (1:2000 in 5% dry milk).

RESULTS

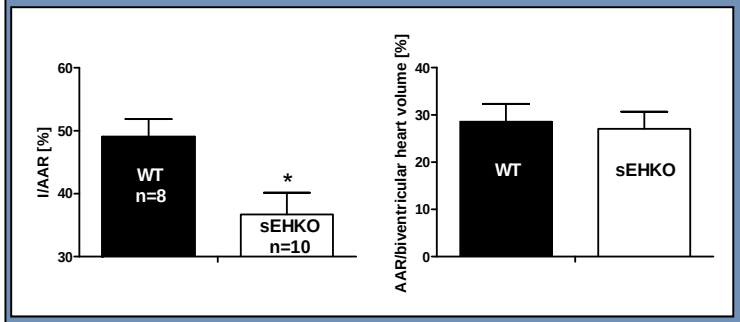


Figure 1: Effect of sEH depletion on infarct size
Myocardial infarct size data expressed as a percent of area at risk (I/AAR) are shown (A). I/AAR was reduced in sEHKO mice compared to wild-type (WT) . (B) AAR as percent of biventricular volume was similar in both groups.

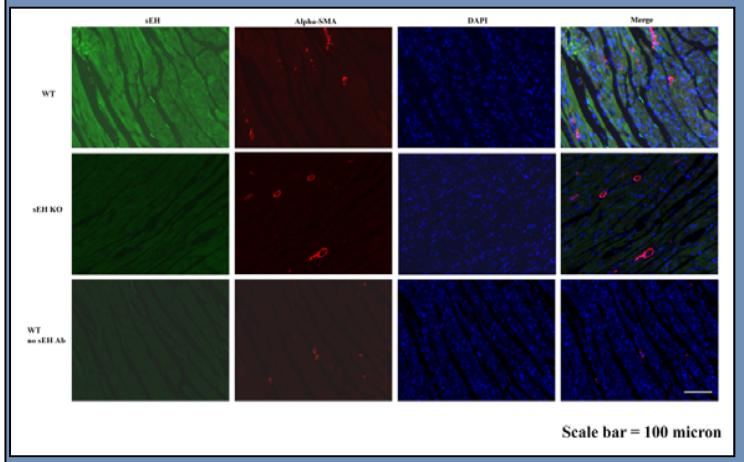


Figure 2: Immunohistochemistry for sEH expression in LV tissue
Shown are immunoreactivity for sEH, α-SMA (myofibroblast) and DAPI (nucleus) in wildt-ype (WT) and sEHKO tissue from left ventricle; bottom row shows immunoreactivity without primary AB.

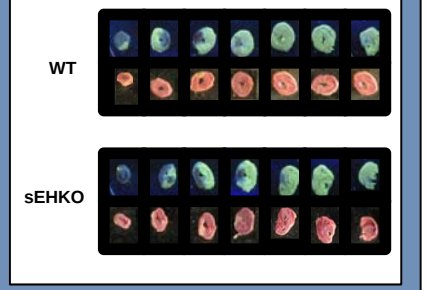


Figure 2: Images of wild-type (WT) and sEHKO hearts
Top row: fluorescent images for AAR analysis
Bottom row: TTC staining for infarct size analysis

	sEHKO		Wild Type	
Body Weight, g	24.8±0.8		28.3±1.0	
Biventricular Weight, mg	115.5±2.6		113.8±4.8	
	Heart Rate [BPM]	Temperature [°C]	Heart Rate [BPM]	Temperature [°C]
Pre-occlusion	535±16**	37.1±0.1	619±12	37.0±0.08
15min after occlusion	534±18	37.0±0.1	577±26	36.9±0.04
5min after reperfusion	501±17	37.0±0.04	546±20	37.0±0.04
30min after reperfusion	492±11*	37.0±0.03	560±14	36.9±0.04
60min after reperfusion	511±15	37.0±0.05	540±10	37.0±0.04
90min after reperfusion	525±12	37.0±0.08	570±12	37.0±0.07
120min after reperfusion	555±15	37.0±0.05	581±9	36.9±0.03

Figure 3: Physiological parameter WT and sEHKO mice
(mean ± SEM; * p<0.05 WT versus sEHKO)

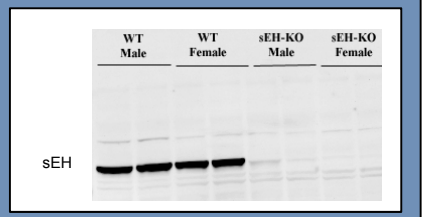


Figure 4: sEH expression via Western Blot
sEH is absent in LV tissue from sEHKO mice in both sexes

CONCLUSION

- sEH inactivation by targeted gene deletion is cardioprotective.
- Enhancing endogenous EET level during and after myocardial ischemia may present a novel pharmacological therapy against myocardial ischemia-reperfusion injury.

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