

Polymorphisms in the Human Soluble Epoxide Hydrolase Gene *EPHX2* are Linked to Cardiomyocyte Survival Following Oxygen and Substrate Deprivation

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ABSTRACT # 479.9

Epoxyeicosatrienoic acids (EETs) protect against myocardial ischemic injury in vivo. *EPHX2* encodes soluble epoxide hydrolase (sEH), the enzyme that breaks down EETs. We tested the hypothesis that *EPHX2* polymorphisms alter cardiomyocyte survival after simulated ischemia. Cultured neonatal murine cardiomyocytes were transduced with five human *EPHX2* variants linked to protein transduction domain TAT. Cultures were subjected to 1.5h of oxygen & glucose deprivation followed by 3h re-oxygenation, with or without sEH substrate 14,15-EET. Cell death was assessed by propidium iodide (% of all cells, mean±SEM, n=5-9 replicates). Successful transduction was confirmed by immunoblot. All mutants reduced cell death compared to the human wild-type *EPHX2* (rh-sEH WT, 63±1%), but only the Lys55Arg and Arg287Gln mutations were different from untransduced controls (54±1% & 45±1% vs. 58±1%, p<0.05 & p<0.001). The Arg287Gln mutation was previously shown to exhibit reduced hydrolase activity. Excess 14,15-EET improved cell survival in all polymorphisms tested except for Arg287Gln (45±1% vs. 45±1%). These data may explain genetic variability in sensitivity to ischemic injury. Support: VA Merit Review 317 (DMVV) and RO1 NS44313 (NJA).

BACKGROUND

The arachidonic acid (AA) derivatives epoxyeicosatrienoic acids (EETs) have anti-inflammatory and anti-thrombotic effects, are coronary vasodilators, and reduce myocardial ischemic injury. The biological activity of EETs is terminated by hydration into less active dihydroeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). More than 10 genetic variants of the *EPHX2* gene encoding soluble epoxide hydrolase in humans have been identified and these polymorphisms have been implicated in susceptibility to cardiovascular disease (1,2). Experimental studies showed that these variants influence the activity of the resulting sEH enzyme (3). Some genetic variants alter the susceptibility to oxygen-glucose deprivation induced death in cultured neurons (4).

HYPOTHESIS

Single nucleotide polymorphisms of the *EPHX2* gene alter cardiomyocyte survival after simulated ischemia

METHOD

Animals were allowed access to phytoestrogen-free food and water ad libitum. With local IACUC approval, all animals 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).

Cell Isolation and Culture

- Postnatal Day 7-8 C57BL/6 mice
- 3-4 hearts from same-gender mice
- Dissociation with 0.625% wt/vol trypsin in Ca²⁺ and Mg²⁺ free HBSS
- Centrifugation and re-suspension in FBS-M199
- Supplementation with estrogen-free 15% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µM cytosine arabinoside,
- Plated at 37°C for 1 hr under a water-saturated atmosphere of 5% CO₂-95% O₂
- Suspended cells were collected and plated at 1.0 × 10⁵ cells/cm²
- incubated as above for 24 hr

TAT-hsEH Fusion Protein Expression and Purification

Six TAT-hsEH fusion proteins, including one wild-type and five *EPHX2* mutants (Lys55Arg, Arg103Cys, Cys154Tyr, Arg287Gln, and Arg103Cys/Arg287Gln), were expressed and purified as previously reported by Koerner et al. (4).

Transduction of TAT-hsEH Fusion Proteins into Cardiomyocytes.

TAT-hsEH fusion proteins were added to the medium of cultured cardiomyocytes to the final concentration of 100 nM. The cells were incubated at 37°C for 24 hours before the onset of oxygen-glucose deprivation.

Oxygen-Glucose Deprivation (OGD)

- Custom-made Plexiglas hypoxia chamber
- OGD 100% N₂ at 37 °C and glucose-free medium (MEM/HBSS)
- Reoxygenation: in 21% O₂ (5% CO₂) and glucose-replete FBS-M199 medium

Determination of Cell Viability

- Cell death assessment via propidium iodide (PI, 5 µM)
- >300 cells/sample

Western blot.

- 4-20% linear gradient SDS-polyacrylamide gels (Bio-Rad), transfer to PVDF membrane
- Incubation overnight at 4°C with primary rabbit anti-sEH antibody (Santa Cruz, 1:2000 in 5% dry milk)
- enhanced chemiluminescence (ECL-plus Western blotting detection kit, Amersham) (1:2000 in 5% dry milk)

14,15-DHET ELISA

- TAT-hsEH (wild-type; Lys55Arg; Arg287Gln) transduced cardiomyocytes were spiked with 14,15-EET (1 µM) for 4.5 hours
- 14,15 DHET production measured with 14,15-DHET immunoassay ELISA

RESULTS

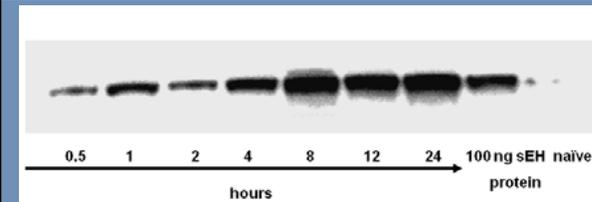


Figure 1: Transduction of TATsEH fusion protein into cultured cardiomyocytes is highly efficient.

Cardiomyocytes were incubated with 100 nM wild-type TAT-hsEH fusion protein and lysed at different time points. Immunoreactivity for sEH in the cell lysate increases over the first 8 hours and remains stable for 24 hours.

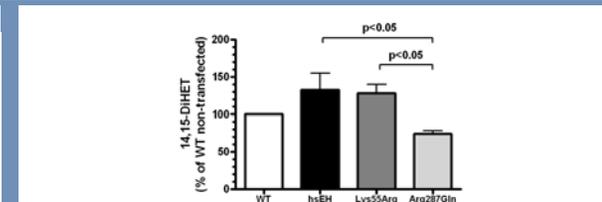


Figure 2: 14,15-DHET production is reduced in Arg287Gln variant.

Epoxide hydrolase activity in cardiomyocytes was measured as production of DHET after spiking with 1 µM 14,15-EET. Shown is the relative production of 14,15-DHET for human wild-type sEH (hsEH WT) and two SNPs compared to naïve murine cardiomyocytes (mean±SEM; n=3).

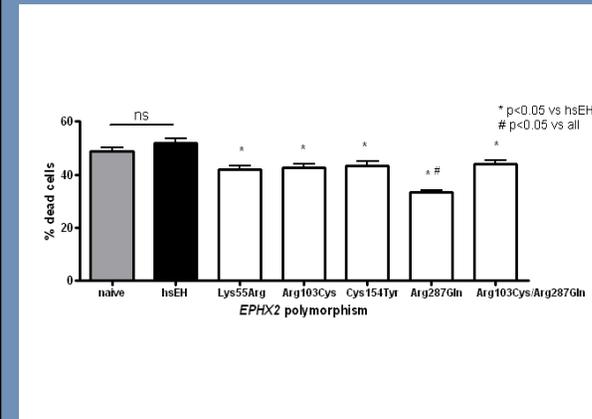


Figure 3: *EPHX2* polymorphisms affect ischemic tolerance against OGD in isolated cardiomyocytes in vitro. Shown is the percentage of dead cells after 3 h of reoxygenation corrected to oxygenated control. The Arg287Gln variant of sEH improved cell survival compared to all tested mutations and human wildtype sEH (hsEH) (n=5, mean ± SEM).

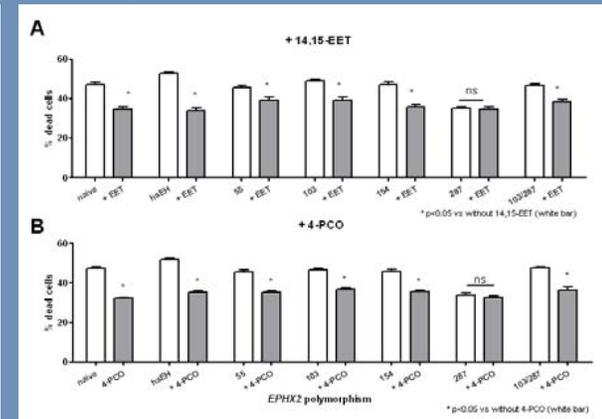


Figure 4: Ischemic tolerance of cardiomyocytes against OGD increases after inhibition of sEH activity or 14,15-EET oversupply.

Shown is the percentage of dead cells corrected to oxygenated control for hsEH, all SNPs and naïve cardiomyocytes tested. **A.** Excess 14,15-EET (1 µM), the substrate of sEH, improved cell survival significantly in all groups tested except the Arg287Gln mutation. **B.** Inhibition of sEH improved cell survival significantly in all groups tested except the Arg287Gln mutation, tested. (n=4 replica, mean ± SEM).

CONCLUSION

- All *EPHX2* variants reduce cardiomyocyte death after simulated in-vitro ischemia.
- Cardiomyocytes transduced with the Arg287Gln variant have reduced epoxide hydrolase activity and improved survival after simulated ischemia.
- Sensitivity to ischemic myocardial injury may therefore partly be due to genetic variability in the human sEH gene

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