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Protective actions of epoxyeicosatrienoic acid: Dual targeting of cardiovascular PI3K and K_{ATP} channels

Sreedhar Bodiga¹, Rong Zhang^{1,8}, Dexter E. Jacobs¹, Brandon T. Larsen², Akihito Tampo³, Vijay L. Manthathi⁶, Wai-Meng Kwok^{2,3}, Darryl C. Zeldin⁷, John R. Falck⁶, David D. Gutterman^{2,4,5}, Elizabeth R. Jacobs¹, and Meetha M. Medhora¹

¹ Pulmonary and Critical Care Medicine, Medical College of Wisconsin, Milwaukee, WI

² Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI

³ Anesthesiology, Medical College of Wisconsin, Milwaukee, WI

⁴ Cardiovascular Medicine, Medical College of Wisconsin, Milwaukee, WI

⁵ Veteran Affairs Medical Center, Milwaukee, WI

⁶ Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX

⁷ Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC

⁸ Department of Pharmacology, College of Pharmacy, Harbin Medical University, Harbin, China 150086

Abstract

Epoxyeicosatrienoic acid(s) (EETs) have been shown to protect cardiovascular tissue against apoptosis dependent on activation of targets such as ATP-sensitive K⁺ (K_{ATP}) channels (sarcolemmal and mitochondrial), calcium-activated K⁺ channels, extracellular signal-regulated kinase or phosphoinositide 3-kinase (PI3K). We tested if EETs protect human atrial tissue *ex vivo* from hypoxia/reoxygenation (H/R) injury, and compared our results with myocardium from two rodent species, rats and mice. EETs reduced myocardial caspase 3 activity in all three species and protected against loss of mitochondrial membrane potential in primary cultures of neonatal rat ventricular myocytes submitted to H/R. In addition, EETs protected mouse pulmonary arteries *ex vivo* exposed to H/R. Myocardium and pulmonary arteries from genetically engineered mice having elevated plasma levels of EETs (*Ephx2*^{-/-}) exhibited protection from H/R-induced injury over that of wild type controls, suggesting that endogenously produced EETs may have pro-survival effects. Electrophysiological studies in myocytes demonstrated that EETs can stimulate K_{ATP} currents in the absence of PI3K. Similarly, activation of PI3K/Akt occurred in the presence of the K_{ATP} channel blocker glibenclamide. Based upon loss of EETs protection in the presence of either wortmannin (a PI3K inhibitor) or glibenclamide, simultaneous activation of at least 2 pathways, PI3K and K_{ATP} channels respectively, appears to be required for protection. In conclusion, we demonstrate that exogenous and endogenous EETs have powerful pro-survival effects in cardiovascular tissues

Corresponding author (to whom the proofs should be sent): Dr. Sreedhar Bodiga, Cardiovascular Center, MEB M4780, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, Tel.: +414-456-4168, Fax: +414-456-6515, E-mail: sbodiga@mcw.edu.

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including diseased human myocardium, mediated by activation of not only one but at least two pathways, PI3K and K_{ATP} channels.

Keywords

human myocardium; pulmonary artery; hypoxia/reoxygenation; mitochondrial membrane potential; epoxide hydrolase; lactate dehydrogenase release

1. Introduction

Cardiovascular disease is the most common cause of mortality in the western world. Localized hypoxic injuries cause cell death that are associated with a number of pathologies in the heart and lungs, including myocardial infarction, transplant injury, acute lung injury and acute respiratory distress syndrome. Cardiomyocytes from human and rodent hearts undergo apoptosis following ischemia/reperfusion [1,2]. Inhibition of cell death reduces the infarct size in rats [3].

Recently, a set of epoxide derivatives of the polyunsaturated fatty acid arachidonic acid, known as epoxyeicosatrienoic acids (EETs¹), have been reported to exhibit potent anti-apoptotic properties [4–7]. Arachidonic acid is a rate limiting substrate for a number of enzymes including cyclooxygenases, lipoxygenases and cytochrome P450 (CYP). CYP monooxygenases catalyze the production of 4 regioisomers of EET, viz.: 14,15-, 11,12-, 8,9- or 5,6-EET. EETs can in turn be hydrated to potentially less active products, dihydroxyeicosatrienoic acids (DHETs), by two forms of epoxide hydrolase (EH): microsomal and soluble [8,9]. Previous studies have demonstrated that unlike EETs, DHETs are not effective against apoptosis of human endothelial cells [4]. Therefore inhibiting the hydration of EETs by EH might increase protection of vascular tissue [10]. EETs activate a number of ion channels including calcium-sensitive K^+ channels (BK_{Ca}) [11,12], ATP-sensitive potassium channels (K_{ATP}) [13] and inhibit cardiac L-type calcium channels [14]. More interestingly, exogenous and endogenous EETs have been implicated in cardioprotective mechanisms that reduce ischemia/reperfusion (IR) injury in rodent and large animal (dog) models [15–17]. These reports have described the effects of EETs to be dependent on stimulation of K_{ATP} channels (sarcolemmal and mitochondrial), based on the findings that pharmacological inhibition of the channels blocked protection of the heart by EETs. In addition, inhibitors of calcium-sensitive K^+ channels, extracellular signal-regulated kinase, phosphoinositide 3-kinase (PI3K) and free radical scavengers also block EET-induced protection against IR [15,16,18,19]. It is not known if EETs elicit the same protective response in human heart and if there is synergy or cross talk between the multiple intracellular pathways that have been implicated in protection.

Previously we tested EETs on cultured myocytes from 2 rodent species, rats and mice, to observe the molecular basis of their anti-apoptotic action from hypoxia/reoxygenation (H/R) injury [6,16]. In the current study we make 3 additional contributions to advance this field: (i) We describe that EETs protect human myocardium by mechanisms similar to those reported in rodents (ii) we introduce a new concept, that pulmonary arteries (PAs) can be preconditioned

¹Abbreviations: EETs, Epoxyeicosatrienoic acids; CYP, Cytochrome P450; DHETs, Dihydroxyeicosatrienoic acids; EH, Epoxide hydrolase; K_{ATP} , ATP-sensitive K^+ channels; IR, Ischemia reperfusion; PI3K, phosphoinositide 3-kinase; H/R, Hypoxia/Reoxygenation; PA, Pulmonary artery; DPBS, Dulbecco's phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; LDH, lactate dehydrogenase; MSPPOH, methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide; MTT, 3-[4,5-dimethylthiazol-2-y]-2,5 diphenyltetrazolium bromide; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; TMRE, tetramethyl rhodamine ethyl ester; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; $\Delta\psi_m$, mitochondrial inner membrane potential; HMR-1098, 1-15-12-(5-chloro-o-anisamido) ethyl-methoxyphenyl)sulfonyl-3-methylthiourea; 5-HD, 5-hydroxydecanoic acid; WT, Wortmannin; Glib, Glibenclamide.

by EETs (iii) we suggest a new model of protection by EET, by simultaneous activation of at least two major signaling pathways (PI3K/Akt and K_{ATP} channels) in parallel as opposed to series, to protect cardiovascular tissue from cell death.

2. Materials and Methods

2.1. Preparation of human myocardium

Human right atrial appendages were obtained as freshly discarded surgical specimens from patients undergoing cardiopulmonary bypass procedures. The specimens were collected in cardioplegic solution at 4-5°C [20]. The atrial appendage was cut manually with a sharp blade to pieces weighing 5-10 mg, in preparation for exposure to H/R (described later). Demographic data and diagnoses of the subjects were obtained from hospital records at the time of surgery. All identifying information which would allow links to individual patients was removed before provision to investigators in the basic science laboratories. These investigations conform to the principles outlined in the Declaration of Helsinki and were approved by Institutional Review Board.

2.2. Preparation of rat/mouse myocardium

All animal protocols were reviewed and approved by IACUC (Institutional Animal Care and Use Committee). Rats (WAG/Rij/MCW), an inbred strain derived from the Wistar background, were maintained at the Medical College of Wisconsin [21]. Animals of both sexes ranging in age from 8-30 weeks (200-300 gm) were used. Mice (C57B16) were purchased from Jackson laboratories and were 6-8 weeks old. Mice with targeted disruption of the *Ephx2* gene [18,22] and matched wild type controls were obtained from the laboratory of Dr. D. C. Zeldin (NIEHS). Routine genotyping was performed as described in the supplementary material.

Animals were anesthetized with pentobarbital sodium (60 mg/kg *i.p.*). Hearts and lungs were rapidly harvested through a median sternotomy, and immersed in phosphate buffered saline (Sigma Chemicals, St Louis, MO). Ventricular or atrial tissue were trimmed to uniform size (~1 mm diameter, 6-7 mm long).

2.3. Preparation of mouse PAs ex vivo

Lungs from 6-8wk-old C57B16 mice were carefully dissected under a microscope. PA rings were isolated, cleared off excess connective tissue and cut into fragments (1-3 mm in length).

2.4. Preparation of fresh adult cardiac ventricular myocytes for electrophysiology

The methods used to isolate ventricular myocytes of adult rats was similar to that previously described [23](for details see supplementary material). Hearts were removed from anesthetized rats and perfused with 2.5 U/ml heparin at pH 7.23, collagenase (Type II, Invitrogen, Carlsberg, CA) and protease (Type XIV, Sigma) for 15-20 minutes. The ventricles were minced and the resulting myocytes were harvested. Myocytes with clear striation patterns were used for the patch clamp studies within 10 hours of harvesting.

2.5. Primary culture of neonatal rat ventricular myocytes

Cardiac ventricular myocytes were prepared from 1 to 2-day-old Sprague-Dawley rats and cultured as described [6,24] (see supplementary materials for details). Hearts were digested with trypsin and the dissociated cells were preplated for 1 h to select the non-adherent myocytes. These cells (>90% cardiac myocytes) were cultured in the presence bromodeoxyuridine (0.1 mM) for 3 days to inhibit fibroblast growth.

2.6. H/R of myocardium and PAs

Resected myocardium (total 30-50 mg pooled tissue) or PAs were placed in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Cat.No.10567-022), 1% penicillin and streptomycin, 0.01% bovine serum albumin (BSA, Sigma, low endotoxin and fatty acid grade) and 10 mM 2-deoxyglucose, D3179, Sigma). Tissues were pretreated for 30 min with inhibitors of PI3K (wortmannin, 200 nM) or K_{ATP} channels (glibenclamide, 10 μ M) before a one time addition of vehicle or EET and then subjected to H/R. The tissues were maintained at 37°C under an atmosphere of 95% N₂ and 5% CO₂ (hypoxia) or 95% air and 5% CO₂ (normoxia) for 8 hours, followed by reoxygenation for 16 hours. The oxygen content (FiO₂) during hypoxia was continuously monitored to be below 1% (Pro-Ox 110, Biospherix Ltd, Redfield, NY). Reagents and inhibitors were added as described in each experiment at the following concentrations: 200 nM wortmannin (ALX-350-020-M005, Alexis Biochemicals), 10 μ M glibenclamide (356310, Calbiochem), 100 μ M pinacidil monohydrate (P154, Sigma), 100 μ M diazoxide (D9035, Sigma) and 300 nM 11,12-EET (1 μ M 11,12-EET for PAs only), 300 nM 14,15-EET, 10 μ M 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE, [25,²⁶] and 20 μ M methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide, (MSPPOH, an inhibitor of endogenous EET biosynthesis [12,^{27,28}] provided by Dr. John R. Falck. Matched volume of vehicle (ethanol and/or dimethyl sulfoxide) were included in controls at a final concentration < 0.01% (vol./vol.).

2.7. Tissue viability by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH)-release

Myocardium or pooled pulmonary artery ring samples were incubated in 0.5 mL of MTT solution (1.25 mg/mL) for 2 h at 37°C, rinsed with PBS, and treated with 2 mL DMSO and incubated at room temperature for another 2 h. The absorbance of 1mL of the solution was measured at 550 nm and normalized to the wet weight of the sample [6] (for details see supplementary material).

LDH released into the culture medium was used as an indicator of membrane damage. It was measured spectrophotometrically (at a wavelength of 340 nm) according to the manufacturer's instructions (Diagnostic Chemicals Limited, Oxford, CT). Enzymatic activity was expressed as percent change from control.

2.8. Caspase-3 assay

The tissues were processed for the caspase-3 colorimetric assay as described by the manufacturer (BF3100, R&D Systems) [29](for details see supplementary material) and results were normalized for equal protein content.

2.9. TUNEL assay

In situ nick-end labeling (TUNEL) of fragmented DNA was performed on paraffin embedded sections (4-5 μ m) of rat myocardium using the manufacturer's protocol (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101, Chemicon International). The percentage of positive apoptotic myocytes was calculated after brown and green nuclei were counted by an operator who was blinded to the treatments of each sample. Values represent the mean $\pm$ standard error of the mean (SEM) from at least 5 different sections (see supplementary materials for details).

2.10. Protein electrophoresis, transfer and immunostaining

The samples were treated under normoxia or H/R with or without 11,12-EET, wortmannin or glibenclamide as described in each experiment. Equal amounts of protein (50 μ g) from each sample were probed by Western blotting. Primary antibodies for phospho-Akt (p-Akt) and Akt

were obtained from Cell Signaling Technology, #9271, #9272) (see supplementary material for details).

2.11. Electrophysiology

Myocytes were placed in an external bath solution containing 5 mM KCl, 132 mM N-methyl-D-glucamine, 1 mM CaCl₂, 2 mM MgCl₂, 5 mM 4-aminopyridine, and 10 mM HEPES with pH adjusted to 7.4 with HCl. The pipette solution contained 60 mM K-glutamate, 50 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.05 mM K₂-ATP, 11 mM EGTA, and 10 mM HEPES with pH adjusted to 7.4 with KOH. ATP-sensitive potassium current, I_{KATP}, was recorded using the whole-cell configuration of the patch clamp technique [23]. Whole cell I_K was monitored during 100-ms test pulses from -120 to +60 mV from a holding potential of -40 mV. For more details see supplementary material. Data acquisition and analysis were conducted using the pClamp software package version 9.2 (Axon Instruments). Additional analyses were performed on Origin version 7 (OriginLab, Northampton, MA).

2.12. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$) in neonatal rat cardiac myocytes

Primary cultures of neonatal rat cardiac myocytes were serum-starved overnight. Myocytes were then treated with 300 nM EET, and inhibitors/agonists (as described in each experiment), in serum-free DMEM containing 0.01% BSA for 30 min. Cells were incubated with 50 nM tetramethyl rhodamine ethyl ester (TMRE) for 15 min at 37°C before addition of 100 μ M hydrogen peroxide (H₂O₂) [30]. Time-lapse fluorescence microscopy was started at 5 min intervals. TMRE was excited at 475 nm and the fluorescence image was collected above 520 nm after passage through a 505 nm dichroic mirror. Twenty cells were randomly selected in each scan and red fluorescence intensity was sequentially captured.

In these experiments, primary cultures of neonatal rat cardiac myocytes were exposed to hypoxia (<1% oxygen) for 8h, followed by reoxygenation (normoxia) for 16 h. These cells were then loaded with 10 μ M 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Molecular Probes, Inc., Eugene, OR, USA) in DMEM at 37°C for 15 min and washed twice with DMEM. JC-1 is a lipophilic and cationic dye that exhibits potential-dependent accumulation in negatively charged mitochondria. The cells were visualized under a fluorescent microscope equipped with red (595 nm) and green (535 nm) emission filters. Fluorescent images were taken with a CCD camera, and analyzed offline with Image-J software (NIH). At low $\Delta\Psi_m$, JC-1 exists mainly in a monomeric form, which emits green fluorescence. At high $\Delta\Psi_m$ JC-1 forms aggregates called "J" complexes, which emit red fluorescence. Thus, a reduction in the ratio of red to green fluorescence indicates a fall in $\Delta\Psi_m$.

2.13. Statistical Analysis

Data are presented as mean $\pm$ SEM. The differences between groups were measured by one-way ANOVA followed by Holm-Sidak's, Tukey's or Fischer's *post hoc* tests using SigmaStat version 3.1. *P*<0.05 between groups was considered statistically significant.

3. Results

3.1. Cell death by H/R in human, rat and mouse myocardium and the protective effect of exogenous EET

Human right atrial appendages were obtained from 16 patients. Patient demographics are summarized in Table 1. Tissue viability was measured as MTT reduction by living cells to a colored product (formazan). Survival decreased by 50% following H/R (8/16 hours) as

compared to aerobically incubated myocardium (normoxia) (Figure 1A). However, addition of 300 nM 11,12-EET before H/R significantly increased tissue survival to 80%. We also assessed caspase-3 activity as an indicator of apoptosis. Caspase-3 activity, increased by over 100% after H/R, whereas treatment with 11,12-EET significantly lowered this value (Figure 1B).

Since 94% of the tissues were obtained from patients with cardiac or cardiovascular disease, the human studies were supplemented by parallel investigations with healthy rodent myocardium from two species, rat and mouse. MTT reduction in rat ventricular myocardium shown in Figure 2A support our observations with the human atrial tissue (Figure 1A), demonstrating that tissue viability was decreased by H/R and that 11,12-EET significantly improved survival. Caspase-3 activity was increased to a similar extent by H/R in both rat and mouse ventricular myocardium, and 11,12-EET (300 nM) prevented this increase (Figures 2B and 2C). Mouse atrial myocardium had a similar response to H/R and was protected by 11,12-EET (Figure 2D).

We tested for apoptosis using a specific assay, TUNEL (Figure 3 and Table 2). Rat myocardium was subject to H/R in the presence of 1 μ M 11,12-EET or vehicle. After incubation for 24 hours under normoxia, approximately 50% of nuclei were apoptotic (stained brown, see dotted arrows) as compared to uninjured nuclei (stained green, see solid arrows). After H/R, the apoptotic index increased to 0.8, but this effect was reversed in the presence of exogenous 11,12-EET (300 nM) (see Table 2).

3.2. Protective effect of endogenous EET(s)

To address the physiological relevance of endogenous EETs, we tested myocardium from a knockout strain of mice (*Ephx2*^{-/-}) defective in expression of the enzyme sEH. Hydration of EETs to less active diols is diminished in this strain resulting in higher circulating levels of EET[10,^{17,31,32}]. Whereas the wild type myocardium showed a significant rise (120%) in caspase-3 activity after H/R, the *Ephx2*^{-/-} derived myocardium showed only a minor increase upon exposure to H/R (Figure 4A).

Cellular injury by H/R in the wild type mouse myocardium was confirmed by two other assays, LDH release and MTT reduction (Figure 4B). Both indicators reported increase in injury after H/R. In addition there was over 3 fold protection in the *Ephx2*^{-/-} myocardium (12%) as compared to the wild type (45%) that was observed by MTT reduction.

To complement the gain-of-function seen with the *Ephx2*^{-/-} mice, we employed a loss-of-function strategy in wild type mouse myocardium using two mechanistically different agents: the EET antagonist, 14,15-EEZE, [25,²⁶] and the P450 epoxygenase inhibitor, *N*-methanesulfonyl-6-(2-proparyloxyphenyl)hexanamide (MSPPOH) [27,²⁸]. 14,15-EEZE alone did not lower caspase-3 activity after H/R, but blocked protection by 11,12-EET. Inhibition of CYP epoxygenase(s) with MSPPOH did not significantly change caspase-3 activity compared to H/R. Adding back exogenous EET to the MSPPOH-treated samples was able to attenuate the increase in caspase-3 activity after H/R caused by MSPPOH (Figure 4C).

3.3. PI3K and K_{ATP} channels mediate the protective effect of EET

Cardiac tissue was subjected to H/R in the presence of wortmannin (200 nM), an inhibitor of PI3K, or glibenclamide (1 μ M), a non-specific K_{ATP} channel blocker (Figures 5A-D). Neither wortmannin nor glibenclamide alone significantly altered MTT reduction (Figure 5A) or caspase-3 activity (Figure 5B) in human myocardium during H/R. Co-application of wortmannin and glibenclamide did not show any further decrease in MTT reduction (Figure 5A) or increase in caspase-3 activity (Figure 5B). However, either alone or both together

completely abolished the protective effect of EET in human (Figure 5A&B) and rat myocardium (Figure 5C). Similar to changes in caspase-3 activity, both wortmannin and glibenclamide increased the apoptotic index (by TUNEL assay) in rat myocardium subjected to H/R, even in the presence of 11,12-EET (Table 2).

We used alternate openers of K_{ATP} channels, pinacidil and diazoxide to mimic the protective effects of EET in human myocardium. These drugs prevented the increase in caspase-3 activity after H/R (Figures 5D), though at concentrations much higher than EET (100 μ M pinacidil, 100 μ M diazoxide versus 300 nM 11,12-EET). Wortmannin, a PI3K inhibitor completely reversed this protection.

3.4 EET preconditions PAs ex vivo

Mouse PAs were carefully dissected and cultured under normoxia or H/R in the presence of 1 μ M 11,12-EET or vehicle. As observed with myocardium, caspase-3 activity was doubled by H/R but not in the presence of EET (Figure 6A). Inhibitors of PI3K or K_{ATP} channels reversed the protective effect of EET. Additionally, PAs from *Ephx2*^{-/-} mice resisted the increase in caspase-3 activity during H/R as compared to wild type animals (Figure 6B). Cellular injury by H/R in the wild type mouse PAs was confirmed by two other assays, LDH release and MTT reduction (Figure 6C). There was decreased injury in the *Ephx2*^{-/-} PAs (24%) versus wild type (46%) as observed by MTT reduction.

3.5. EET maintains mitochondrial membrane potential during oxidative stress

Since apoptosis is associated with loss of mitochondrial integrity, we examined the effects of EET on the mitochondrial electrochemical gradient ($\Delta\psi_m$) using the fluorescent indicator TMRE. This is a cationic dye that accumulates in mitochondria and reflects the negative mitochondrial matrix potential under normal conditions, which is lost upon depolarization of the organelle. For uniform monitoring of cell fluorescence, we used monolayers of cultured neonatal cardiomyocytes instead of tissue sections. We have demonstrated that these cells, similar to adult rodent tissue, undergo apoptosis by H/R that is reversed by EETs [6]. Myocytes were treated with 100 μ M H_2O_2 to mimic oxidative stress, a key feature of ischemic damage. There was a progressive loss of fluorescence with time over a period of 90 min with H_2O_2 (Figure 7A, (i)). Pretreatment with 300 nM 11,12-EET for 30 min prior to H_2O_2 exposure prevented the loss of TMRE fluorescence and enabled the visualization of punctuate staining (Figure 7A, (ii)). However, this protective effect of EET was abrogated by pretreatment with wortmannin or glibenclamide and following the application of H_2O_2 (Figure 7A, (iii)). Similar to EET, K_{ATP} agonists, pinacidil and diazoxide prevented the loss of mitochondrial membrane potential after H_2O_2 challenge, but not in the presence of wortmannin (Figure 7A, (iv)). Please see supplementary figure 1 for time-lapse recording of TMRE fluorescence.

These results were confirmed using another indicator of mitochondrial membrane potential, JC-1, and a second form of injury, H/R. When the mitochondria are depolarized, the most distinctive feature of JC-1 is an emission shift from orange to green. In primary cultures of rat neonatal cardiomyocytes, H/R lead to a predominant green staining as compared to the orange staining observed under normoxia (Figure 7B), indicating a reduction in the mitochondrial inner membrane potential. In agreement with experiments using TMRE, EET restored the red/green ratio of mitochondrial staining by JC-1, but not if wortmannin or glibenclamide were present. In addition, pinacidil and diazoxide mimicked the effect of 11,12-EET. These results are graphically represented in Figure 7C.

3.6. PI3K is activated by EET in the presence of glibenclamide

In order to explore activation of PI3K by EET, we followed phosphorylation of Akt in rat myocardium subjected to H/R. Using an antibody specific for the phosphorylated form of this

kinase (p-Akt, ser 473), we measured the changes in p-Akt expression (Figure 8A(i) and (ii)). 11,12-EET significantly increased p-Akt expression in myocardium subjected to H/R, which was blocked by wortmannin. In contrast, glibenclamide, an inhibitor of K_{ATP} channels did not affect p-Akt expression in response to 11,12-EET.

3.7. K_{ATP} channels are activated by EET in the presence of wortmannin, a PI3K inhibitor

The external application of 14,15-EET (1 μ M) increased the outward whole-cell current amplitude relative to vehicle control (Figure 8B, (i)) in isolated rat cardiac myocytes. EET induced increase in whole cell current was blocked by 1 μ M glibenclamide. Thus, the current elicited by 14,15-EET was identified as largely I_{KATP} . As demonstrated in Figure 8B, (ii), the subtracted glibenclamide-sensitive current was greater than the subtracted 14,15-EET-sensitive current. This suggests that under our recording conditions, there is a basal level of I_{KATP} activation. To investigate the effect of PI-3 kinase blockade on the 14,15-EET induced I_{KATP} , cells were incubated in the Tyrode solution containing wortmannin (10 μ M) for 30 minutes. Despite this pretreatment, 14,15-EET was able to elicit I_{KATP} which was densely blocked by glibenclamide (Figure 8B, (iii)). This suggested that inhibition of PI3K did not prevent activation of I_{KATP} by 14,15-EET. Figure 8C demonstrates the effect of 14,15-EET on I_{KATP} in another example with the corresponding current-voltage (I-V) relationships. The I-V curves exhibited a rectifying feature at the positive potentials that is characteristic of block of whole-cell I_{KATP} by intracellular Mg^{2+} ions [33, 34]. The results of 14,15-EET and wortmannin on I_{KATP} (glibenclamide sensitive current at +60 mV) are summarized in Figure 8C, (iii).

4. Discussion

In this study, we tested adult myocardium and PAs *ex vivo* for protection by EETs against apoptosis induced by H/R. This approach allowed us to examine the pro-survival action of EET on human heart for the first time. We report that 11,12-EET protected human myocardium including that obtained from patients with cardiovascular disease. This is an important and clinically relevant finding, in that therapies that usually reduce simulated IR in healthy animal models might not be effective in tissue from humans with coronary disease. Rescue by EET of human myocardium was comparable to that observed in myocardium from two other well researched mammalian species, mouse and rat. Mouse hearts and PAs deficient in expression of *Ephx2*, and therefore having elevated levels of EET [18] were markedly protected from apoptosis after H/R, unlike wild type tissues in which H/R induced significant injury measured by three indicators, MTT-reduction, LDH release and caspase 3 activity. The structurally related EET-derivative 14,15-EEZE blunted protection observed in the presence of EET (Figure 4C). The CYP inhibitor MSPPOH did not significantly alter caspase 3 activity, indicating there could be a low level of endogenous EET synthesis under the experimental conditions. As expected, supplementation with exogenous EET restored protection even in the absence of endogenous generation of EET.

Another novel finding is the ability of EETs to maintain mitochondrial membrane potential, an anti-apoptotic action that has not previously been reported. Using two models of injury, the first by adding high concentrations of the oxidant H_2O_2 and the second by H/R (which is widely believed to increase intracellular oxidants), we demonstrated that exogenous EET prevented the loss of mitochondrial membrane potential in cultured myocytes.

To investigate the cellular mechanisms by which EETs induce protection, we tested two molecular pathways that have been proposed to contribute to preconditioning: K_{ATP} channels [13, 15, 16, 18, 19, 26, 35–37] and PI3K [4, 6, 29, 38–40]. Our results indicate that either K_{ATP} or PI3K inhibitors or both negate the protective effects of 11,12-EET in our *ex vivo* models. Importantly, this mechanism is common between humans, mice and rats.

In order to examine if there is cross-talk between the 2 pathways, we evaluated a downstream effector of PI3K, p-Akt, in the presence of glibenclamide. At the end of H/R, the activated state of Akt was not altered suggesting that PI3K remained functional in spite of the blockade on K_{ATP} channels. We also tested the effect of the PI3K inhibitor wortmannin on the ability of EET to open K_{ATP} channels in adult rat myocytes. Since wortmannin did not prevent activation of I_{KATP} by 14,15-EET, the observed cardioprotective effect of EET includes a pathway (PI3K) that is independent of the sarcolemmal K_{ATP} channel. Channel activity (Figures 8B & 8C) was measured with a different regioisomer of EET, 14,15-EET [13]. However, this has comparable protection to 11,12-EET in rat hearts as well as cultured rat and mouse myocytes [6, 15, 16, 18, 36, 37]. Similar to our results with 14,15-EET, 11,12-EET has been demonstrated to open K_{ATP} channels in cardiomyocytes [13, 15, 16, 18, 26, 36, 37]. We show that EET increases K_{ATP} channel currents in myocytes even when PI3K is inhibited with wortmannin.

These results also suggest that PI3K and K_{ATP} channels do not function in series, i.e., activation or inhibition of one pathway does not turn on or shut off the other, since we observed that each pathway remained active while the other was inhibited. It is likely that these pathways act non-redundantly, but in combination, for preconditioning of cardiovascular tissues by EETs as represented by the schematic in Figure 9. Both pathways may work in parallel to complement each other in bringing about protection. We speculate that signaling intermediates from both pathways may interact to preserve mitochondrial integrity (Figure 9) or dock to a common adapter that allosterically modulates survival. We observed that two different agonists of the K_{ATP} channels, pinacidil or diazoxide, also protect cardiomyocytes. These pro-survival stimuli, similar to those manifested by EET, are also blocked by wortmannin, confirming that PI3K must be active in parallel with the K_{ATP} channels to attenuate cell death. In addition both agonists acutely activate PI3K, though they do not alter the state of pAkt after H/R (8/1 hour) as observed by EET (Supplementary Figure 2, A-D). Taken together these results suggest that multiple anti-apoptotic pathways may function in parallel to mitigate programmed cell death or injury (Figure 9). Therefore, we cannot rule out other EET-induced intermediates that have been implicated in protection, one example being the initial burst of reactive oxygen species with the application of EETs and its association with opening of K_{ATP} channels [36].

Subcellular localization of the K_{ATP} channels involved in EET-induced protection remains to be determined. In animal models of IR injury, treatment with 14,15- or 11,12-EET significantly reduced infarct size, an effect that was completely blocked by glibenclamide, 5-hydroxydecanoate (5-HD) and the selective sarcolemmal K_{ATP} channel antagonist, 1-15-12-(5-chloro-*o*-anisamido)ethyl-methoxyphenyl)sulfonyl-3-methylthiourea (HMR-1098) [15, 16, 26, 36]. Interestingly, besides PI3K blockers, cardioprotection observed in *Ephx2*^{-/-} hearts was also completely abolished by glibenclamide, 5-HD that blocks mitochondrial K_{ATP} or paxilline that inhibits calcium-sensitive K^+ channels [15, 18]. It is beyond the scope of the present study to examine all these permutations. EETs open both sarcolemmal and mito K_{ATP} channels [13, 41, 42]. Though pharmacological blockers for mitochondrial K_{ATP} channels are often used, these channels are not yet defined at a molecular level to permit determination of their activated states as we have done for the sarcolemmal channels (Figures 8B & 8C). Mitochondrial ATP-sensitive potassium channels are believed to inhibit apoptosis induced by oxidative stress in cardiac cells [24, 43]. They also attenuate early apoptotic events, such as cytochrome *c* release, mitochondrial membrane depolarization [24, 44, 45] and opening of the mitochondrial permeability transition pore (PTP) that results in apoptosis. Ion balance in mitochondria plays a critical role in regulating the PTP [46]. We show that EET preserves mitochondrial depolarization by H_2O_2 or H/R (Figures 7A-C).

EETs activate PI3K/Akt and recent studies have suggested that Akt inhibits apoptosis at a premitochondrial level because it inhibits cytochrome *c* release and alteration of mitochondrial membrane potential [47–50]. However the mechanism is still obscure. Activation of the PI3K/

Akt pathway has been implicated in various ischemic preconditioning models [6,³⁹,⁵¹] and PI3K has emerged as a critical signaling molecule for survival and proliferation.

In summary, we have observed EET-induced protection of ventricular or atrial myocardium against H/R-injury in *ex vivo* cultures of myocardium by 11,12-EET (300 nM) from three species: human, rat and mouse. This beneficial effect occurred in tissue from older humans including those with cardiovascular disease. Protection of myocardium or PAs *ex vivo* by EET required both PI3K and K_{ATP} channels to be functional. Our studies did not support the existence of a linear pathway of PI3K/Akt and K_{ATP} channels acting in series to mediate the beneficial effects of EET. In fact, our results lead us to believe that multiple, intracellular pro-survival systems must act in concert or parallel to regulate preconditioning and protect cardiovascular tissues. EETs are particularly attractive as therapeutic agents, since they are non-immunogenic, naturally formed products, and can rescue tissue injury to diseased human myocardium. Our findings, as well as those of others, suggest that sEH inhibitors may provide a therapeutic option because they may enhance bioavailability of endogenous EET to the myocardium, to promote survival after H/R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Protection by EET of human myocardium from H/R induced cell death. Human atrial tissue *ex vivo* from patients with cardiovascular disease (see Table 1) were maintained under normoxia (24 h) or H/R (8/16 hours) after pretreatment with vehicle or 11,12-EET (300 nM). Tissues were assayed (see Material and Methods for details) for : (A) reduction of MTT to determine viability after H/R as compared to normoxia (B) caspase-3 activity as a measure of apoptosis. Note decreased MTT reduction and increased caspase-3 activity after H/R, that is significantly attenuated by pretreatment with 11,12-EET. All data are mean $\pm$ SEM from 6-8 experiments. * indicates $p < 0.05$ vs. normoxia (adjusted to 100% in each experiment); ** indicates $p < 0.05$ vs. H/R.

Figure 2.

Protection by EET of rodent myocardium *ex vivo* from H/R induced cell death. Healthy rodent myocardium was maintained under normoxia (24 h) or H/R (8/16 hours) after pretreatment with 11,12-EET (300 nM). Tissues were assayed for: (A) reduction of MTT by rat ventricular myocardium (B) caspase-3 activity in rat ventricular myocardium (C) caspase-3 activity in mouse ventricular myocardium (D) caspase-3 activity in mouse atrial myocardium. Note decreased MTT reduction and increased caspase-3 activity after H/R, that is significantly attenuated by 11,12-EET. All data are mean $\pm$ SEM from 6-8 experiments. * indicates $p < 0.05$ from normoxia (adjusted to 100% in each experiment), ** indicates $p < 0.05$ vs. H/R.

Figure 3.

In situ nick end labeling (TUNEL) of paraffin-embedded rat myocardial sections (4-5 μ m). Rat ventricular myocardium *ex vivo* was maintained under normoxia (24 h) or H/R (8/16 hours). Tissue sections were stained by TUNEL and nuclei with fragmented DNA (brown, also marked by dotted arrows) were counted along with normal nuclei that were stained green (solid arrows). Results from sections after different treatments were computed by an operator blinded to the experimental conditions and recorded in Table 2.

Figure 4.

Protection of mouse myocardium by endogenous EET. Mouse myocardium *ex vivo* was maintained under normoxia (24 h) or H/R (8/16 hours). Tissues were assayed for caspase-3 activity in: (A) ventricular myocardium from wild type (WT) and *Ephx2*^{-/-} mice. Note absence of increase in caspase 3 activity after H/R in *Ephx2*^{-/-} mice; n = 6; **P* < 0.05 compared with wild type (normoxia) (B) LDH release and MTT reduction were increased in myocardium from WT mice. Increased viability by the MTT assay was observed in *Ephx2*^{-/-} mouse myocardium compared to WT after H/R (C) ventricular myocardium following pretreatment with 14,15-EEZE (10 μM) or MSPPOH (20 μM). 14,15-EEZE antagonized the effect of 11,12-EET, whereas MSPPOH did not influence the effect of exogenous EET. All data are mean ± SEM

from 4-6 experiments. * indicates $p < 0.05$ vs. corresponding normoxia (adjusted to 100% in each experiment); ** indicates $p < 0.05$ vs. corresponding H/R.

Figure 5.

EETs protect from H/R by PI3K or K_{ATP} channels. Human or rat myocardium *ex vivo* were maintained under normoxia (24 h) or H/R (8/16 hours) after pretreatment with vehicle (unlabeled), wortmannin (a PI3K inhibitor), or glibenclamide (an inhibitor of K_{ATP} channels) for 30 min, with or without 11,12-EET (300 nM), pinacidil (100 μ M) or diazoxide (100 μ M). Tissues were assayed for: (A) reduction of MTT by human myocardium to determine viability after H/R as compared to normoxia, (B) caspase-3 activity in human myocardium (C) caspase-3 activity in rat ventricular myocardium (D) caspase-3 activity in human myocardium treated with K_{ATP} channel openers pinacidil and diazoxide. Note that wortmannin or glibenclamide abolish the protective effects of EET and the K_{ATP} channel openers. All data are mean $\pm$ SEM from 6-8 experiments. * indicates $p < 0.05$ from normoxia (adjusted to 100% in each experiment), ** indicates $p < 0.05$ vs. H/R.

Figure 6.

Preconditioning of mouse pulmonary arteries *ex vivo* with 11,12-EET. Mouse pulmonary arteries *ex vivo* were maintained under normoxia (24 h) or H/R (8/16 hours) (A) Caspase-3 activity was measured after pretreatment for 30 min with vehicle (unlabeled), wortmannin or glibenclamide, with or without 11,12-EET (300 nM). Note that wortmannin or glibenclamide abolish the protective effects of EET. (B) Caspase-3 activity was measured in pulmonary arteries from wild type or *Ephx2*^{-/-} mice. *Ephx2*^{-/-} mice are dramatically resistant to increase in caspase-3 after H/R observed in WT PAs. (C) LDH release and MTT reduction in PAs of WT and *Ephx2*^{-/-} mice as labeled. Increased cell injury and decreased survival was observed in WT PAs after H/R, and this was attenuated in PAs from *Ephx2*^{-/-} mice. All data are mean $\pm$ SEM from 5-8 experiments. * indicates $p < 0.05$ vs. corresponding normoxia (adjusted to 100% in each experiment); ** indicates $p < 0.05$ vs. corresponding H/R.

Figure 7.

Figure 7A. EET protects cultured myocytes from loss of mitochondrial membrane potential ($\Delta\psi_m$) during injury. Cardiac myocytes were serum deprived for 16 h and treated with wortmannin (200 nM) or glibenclamide (10 μ M) for 30 min. For the next 30 min, the cells were incubated with 11,12-EET (300 nM), pinacidil (10 μ M) and diazoxide (10 μ M) as mentioned in the Materials and Methods. The cells were loaded with the fluorescent indicator TMRE (50 nM) and challenged with H_2O_2 (100 μ M). Time lapse images were captured for 90 min at 5- min intervals. Representative sequential images from selected time points from a set of four individual experiments are shown. Treatments included 100 μ M H_2O_2 and (i) vehicle, 0-90 min (ii) 11,12-EET, 0-90 min (iii-iv) inhibitors and/or K_{ATP} channel openers as labeled, after 90 min of H_2O_2 . Note the gradual loss of TMRE fluorescence (490/510 nm) upon H_2O_2 treatment in the absence of EET, indicating a reversible loss of $\Delta\psi_m$. EET preserves the TMRE fluorescence, though not in the presence of PI3K or K_{ATP} channels inhibitors. Pinacidil and diazoxide also preserved loss of TMRE fluorescence by H_2O_2 .

Figure 7B. JC-1 fluorescence as a measure of mitochondrial membrane potential. The experiment in Figure 7(A) was repeated using a second indicator, JC-1 and a different injury,

H/R. Cells were loaded with JC-1 (10 μ M) for 30 min and viewed under a fluorescence microscope. Representative pictures of each treatment (as labeled) from 4 independent experiments are shown. Note the robust red/green fluorescence under normoxia is attenuated by H/R, but maintained by EET, pinacidil and diazoxide, only in the absence of inhibitors of PI3K or K_{ATP} channels.

Figure 7C. Quantitative analysis of red/green fluorescence (595/535 nm) with JC-1 in neonatal rat cardiac myocytes. Data are mean $\pm$ SEM from 4 independent experiments. * indicates $p < 0.05$ vs. normoxia; ** indicates $p < 0.05$ vs. H/R. Higher ratios indicate normal membrane potential ($\Delta\psi_m$).

Figure 8.

Figure 8A. Activation of PI3K/Akt by 11,12-EET, in the presence of the K_{ATP} channel antagonist, glibenclamide. Rat myocardium was maintained under normoxia (9 h) or H/R (8/1 hours) after pretreatment with vehicle or 11,12-EET (300 nM). Pharmacological inhibitors of PI3K (wortmannin, 200 nM) or K_{ATP} channels (glibenclamide, 10 μ M) were included as labeled. (i) Representative Western blot of cell lysates probed with phospho-Akt-Ser473 (upper panel). The blots were stripped and reprobed with Akt (lower panel) (ii) Phospho-Akt and Akt band densities were analyzed by densitometry and the mean $\pm$ SEM from four independent experiments were used to calculate the ratio of phospho-Akt/Akt. * indicates $p < 0.05$ vs. normoxia; ** indicates $p < 0.05$ vs. H/R.

Figure 8B. Identification of the 14,15-EET activated current. (i) Whole-cell current was recorded from isolated ventricular myocytes in the presence of vehicle or 14,15-EET (1 μ M). The 14,15-EET activated current was blocked by 1 μ M glibenclamide (Glib). Consequently, the Glib-sensitive current as identified as I_{KATP} . (ii) 14,15-EET sensitive and Glib sensitive currents were obtained from subtracted traces: 14,15-EET – Control traces, and 14,15-EET – Glib traces, respectively. (iii) Following a 30-minute incubation in 10 μ M wortmannin, currents from myocytes were recorded in the presence of 1 μ M 14,15-EET. 14,15-EET activated a glibenclamide-sensitive current despite prior exposure to wortmannin.

Figure 8C. Effects of 14,15-EET on I_{KATP} . Whole-cell current was recorded from isolated ventricular myocytes in the presence of vehicle or 14,15-EET (1 μ M). The current traces and the corresponding current-voltage relationships are shown. (i) demonstrates the activation of I_{KATP} by 14,15-EET. (ii) demonstrates a similar activation of I_{KATP} following 30 min incubation with wortmannin (10 μ M). Note the characteristic rectification of the I_{KATP} I-V curve at the positive test potentials, indicative of block of current by intracellular Mg^{2+} . A summary of the 14,15-EET and wortmannin (post-WT) effects on I_{KATP} are presented in (iii). I_{KATP} density was monitored at a +60 mV test pulse from a holding potential of -40 mV.

Figure 9.

Schematic depicting model of mechanism of protection of cardiovascular tissues by EETs. These endogenous fatty acids are products of the cytochrome P450 family 2 enzymes (CYP2). They activate at least 2 intracellular signaling systems, the PI3K/Akt pathway and K_{ATP} channels, based on our results (Figure 5-8) and as marked by solid arrows. In addition, they preserve mitochondrial membrane potential from depolarization by injurious oxidants (Figure 7). Inhibition of either pathway, PI3K or K_{ATP} channels abolishes the protective effect of EETs. We speculate (as shown by dotted lines) that this may occur via complementary intermediates that are generated by PI3K and K_{ATP} channels (and probably other signaling systems that are activated by EETs) to act in concert. Therefore multiple parallel signaling pathways such as PI3K and K_{ATP} channels may converge, rather than act in series, to either reverse cellular injury or block cell death.

Table 1

Patient demographics (n=16)

Characteristics	n (%)
Sex, male	9 (56)
Age, years (mean $\pm$ SD	71 $\pm$ 8
Underlying diseases/risk factors	
Coronary artery disease	15 (94)
Hypercholesterolemia	10 (63)
Atrial fibrillation	11 (69)
Hypertension	10 (63)
Valve disease	10 (63)
Diabetes mellitus	9 (56)

Table 2
Apoptotic index in rat myocardium subjected to H/R

11,12-EET (300 nM) reverses increase in apoptotic indices induced by H/R. Fragmented DNA in rat myocardium was labeled by TUNEL (see Material and Methods for details). The number of positive apoptotic myocytes was counted in a blinded manner in nuclei from at least 5 different sections of each treatment. Apoptotic cell index was presented as the ratio of TUNEL-positive nuclei (brown staining)/total number of nuclei (brown+green).

Group	Apoptotic Index
Normoxia (n = 5)	0.54 ^{**} ±0.12
H/R (n = 9)	0.81 [*] ±0.09
H/R+11,12-EET (n = 8)	0.50 ^{**} ±0.10
H/R+ Wortmannin (n = 7)	0.82 ^{**} ±0.11
H/R+ Wortmannin+11,12-EET (n = 6)	0.78 [*] ±0.10
H/R+ Glibenclamide (n = 8)	0.79 [*] ±0.10
H/R+ Glibenclamide + 11,12-EET (n = 7)	0.80 [*] ±0.14

Values represent the mean ± SEM;

* p < 0.05 vs. normoxia and H/R+11,12-EET;

** p<0.05 vs. H/R.