Multiple antiapoptotic targets of the PI3K/Akt survival pathway are activated by epoxyeicosatrienoic acids to protect cardiomyocytes from hypoxia/anoxia

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The regulation of cell survival is crucial to the normal physiology of multicellular organisms. The role of apoptosis in heart disease has been debated, although recent evidence suggests that it contributes to tissue injury in several cardiac disorders including myocardial infarction, atherosclerosis, transplant, myocarditis, and cardiac failure. From 5% to 30% of cardiac myocytes undergo apoptosis in rodent and humans within 16 h of reperfusion (2, 21, 47, 50, 60), and this trend persists for months (up to 0.25% in diseased humans compared with up to 0.002% in controls) (28, 46, 60). In intact animals, apoptosis occurs during myocardial infarction, heart failure, and transplant injuries (13). Genetic approaches using rodents have determined that inhibition of apoptosis in cardiomyocytes reduces infarct size by 50–70% (9, 37, 50). Apoptotic rates as low as 0.023% are sufficient to cause lethal dilated cardiomyopathy in transgenic mice that are engineered to have cardiac-specific expression of caspase-8. This rate of apoptosis is 5- to 10-fold lower than that measured in cardiac tissue from patients with end-stage heart failure (13, 46). A recent finding in a renal proximal tubular epithelial cell line demonstrated that EETs inhibit apoptosis induced by serum withdrawal and etoposide (6, 41). We have reported that EETs inhibit apoptosis induced by the engagement of the death receptor Fas or by serum withdrawal in human vascular endothelial cells cultured in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
from the heart or lung (17, 36). Hence, we were interested to see whether EETs have the same antiapoptotic effect in cardiomyocytes after I/R since myocardial cell death caused by apoptosis is a main feature of this condition.

We investigated four different end points to report whether EETs inhibit cell death/apoptosis in cultured cardiomyocytes, which are subjected to hypoxia and reoxygenation (H/R) to simulate conditions of I/R. We describe for the first time the activation of the enzyme PI3K by EETs in cultured myocytes. We also systematically followed multiple downstream antiapoptotic signals, including phosphorylation of protein kinase B (Akt) and bcl-xl/bcl-2-associated death promotor (BAD), increased intracellular levels of X-linked inhibitor of apoptosis (XIAP), and decreased activity of caspases-9 and -3. Our results support at least five prosurvival effectors that may mediate the EET-induced protection of cardiomyocytes via stimulation of the PI3K/Akt pathway.

MATERIALS AND METHODS

Culture of Neonatal Rat Ventricular Myocytes

Animals were housed in the accredited Biomedical Resource Center. All procedures for animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee. Hearts from 1-day-old Sprague-Dawley neonatal rats were excised, and the ventricular myocardium was cut into small pieces (~2 mm³) in dissociation buffer containing (in mm) 116 NaCl, 20 HEPES, 1 NaH₂PO₄, 5.5 glucose, 5.4 KCl, and 0.8 MgSO₄ and 0.6 ml/l phenol red (pH 7.35) with 0.15 mg/ml collagenase (Worthington II, Lake-wood, NJ) and 0.52 mg/ml pancreatin (Life Technologies, Grand Island, NY) and incubated on a shaker at 37°C for 20 min at 100 rpm. Tissue pieces were allowed to settle, and the supernatant (containing myocytes) was collected, suspended in 1 ml newborn calf serum (GIBCO, Carlsbad, CA), and centrifuged at 1,000 rpm for 6 min. The cell pellet was resuspended in 1 ml newborn calf serum and stored at 37°C. The procedure was repeated until all tissue was digested. The cells were then resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 17% medium 199, 10% fetal bovine serum, 0.5% penicillin-streptomycin, and 20 mm HEPES (pH 7.2) for 2 h. This facilitates the separation of ventricular myocytes from the faster-attaching nonmyocytes. The ventricular myocytes in the supernatant were collected and plated on gelatin-coated dishes. They were cultured in media containing bromodeoxyuridine (5-bromo-2-deoxyuridine) at a final concentration of 0.1 μM. The cells were used for experiments after demonstrating rhythmic contractions (48–72 h).

HL-1 Cells

Cells of HL-1, a cardiac muscle cell line derived from the AT-1 mouse atrial myocyte tumor lineage, were a gift from Dr. William C. Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA) and maintained accordingly as described (11). HL-1 cells were used for experimentation after reaching ~70–80% confluence.

Treatment of Cells

Rat neonatal myocytes were cultured to 70% confluency and then serum starved in basal medium (DMEM + 0.1% BSA) for 24 h for the experiments in Fig. 1. The cells were stimulated with one of four concentrations of 14,15-EET (0.1, 0.3, 1, or 10 μM) or vehicle (ethanol) and lysed as described in Western Blot Analysis. For other experiments, separate groups of neonatal rat ventricular myocytes or mouse atrial HL-1 cells received no intervention (normoxia controls) or were exposed to H/R after pretreatment with two applications of either vehicle (ethanol) or a single regioisomer of EET (1 μM of 14,15-, 11,12-, or 8,9-EET). Exposure to EET was accomplished 16 h before (first application) and just before (second application) H/R. Hypoxia was simulated for 8 h in neonatal myocytes by exposure to 5% CO₂-95% N₂ in an airtight chamber in the presence of serum- and glucose-free DMEM containing 10 mM deoxyglucose to inhibit glycolysis. The levels of oxygen were monitored continuously and were below 2% at all times. Reoxygenation was performed for 16 h in DMEM + 10% FBS except if otherwise mentioned [for PI3K activity or analyses of phospho-Akt (pAkt)]. HL-1 cells were treated similarly except that deoxyglucose was not included in the serum-free medium. In some experiments, the PI3K inhibitor wortmannin (WT) was added 30 min before each application of EET. Control cells that received no intervention or H/R were maintained in DMEM + 10% FBS throughout the duration of the experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The cells (HL-1 and neonatal myocytes) were cultured in 60-mm dishes (plated at a density approximately half million cells) to ~80% confluency and then maintained in either complete medium or switched to basal medium after 42 h. The samples were maintained under normoxia or treated with a single regioisomer of EET or vehicle and subjected to H/R as described in Treatment of Cells. At the end of the incubation period, the survival of the cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in the manufacturer’s protocol (Cat. No. V-13154; Molecular Probes, Eugene, OR). Briefly, the cells were incubated for 3 h in phenol red-free medium containing 0.5% of the yellow mitochondrial dye MTT⁺. We used a slightly higher confluency (~80%) in the MTT protocol since the assay required cell viability for
an additional 3 h of incubation with the substrate after exposure to H/R. The amount of blue formazan dye generated from MTT” was proportional to the number of live cells. The MTT” reaction was terminated by the addition of DMSO to the medium, followed by incubation for 10 min at 37°C. The absorbance was read at 540 nm in a spectrophotometer. The values of the reaction were obtained after the subtraction of matched blanks, and the optical densities of the controls were taken as 100% for comparisons with values for other samples. The readings for the test cells were expressed as percentages of control.

Hoechst Staining

The cells were cultured in six-well plates to 70% confluence, treated with EETs, and subjected to H/R as described for the MTT assay in Treatment of Cells. They were then stained with 1 µl of Hoechst 33342 (5 mg/ml; Cat. No. V-13244, Molecular Probes) in 1 ml basal medium and incubated for 30 min. Stained cells were washed twice with PBS (Sigma, St. Louis, MO) and imaged under a fluorescent microscope (excitation, 350 nm; emission, 460 nm).

Annexin V Binding

Annexin V binds to phosphatidylserine, which appears in the outer leaflet of the plasma membrane in early apoptotic cells. Cells (neonatal myocytes and HL-1 cells) were cultured in 60-mm dishes to 70% confluency and subjected to normoxia or H/R with or without pretreatment with EET as described in Treatment of Cells. The cells were washed with PBS and treated with FITC-labeled annexin V (0.2 µg/ml) for 20 min at room temperature (17). Labeling with FITC-coupled annexin V was performed according to the manufacturer’s protocol (BD Biosciences, San Diego, CA) as previously described (17). The labeled cells (10,000/sample) were analyzed by measuring fluorescence intensity using a FACScan flow cytometer (Becton Dickinson) in conjunction with CellQuest software (BD Biosciences).

Activity of Caspases-3 and -9

Cells (neonatal myocytes and HL-1 cells) were cultured in 60-mm dishes to 70% confluency and subjected to H/R or normoxia with or without pretreatment with EET as described in Treatment of Cells. The cells were collected by centrifugation, washed twice with ice-cold PBS, resuspended in lysis buffer containing (in mM) 5 MgCl2, 1 EGTA, 0.1% Triton X-100, and 25 HEPES (pH 7.5), and stored overnight at −80°C as previously described (28). The release of free 7-amino-4-trifluoromethyl coumarin (AFC) from the synthetic substrate N-acetyl-Asp-Glu-Val-Asp-AFC (50 µM) for caspase-3 and the release of free AFC from the N-acetyl-Leu-Glu-Asp-AFC substrate for caspase-9 at 37°C were determined by fluorescence measurements in a SpectraFluor Plus plate reader after excitation at wavelength of 390 nm. Emission was measured at 535 nm.

Detection of Cleaved Caspase-3 Activity

Cells were treated under normoxia or H/R with or without EET, and cleaved caspase was detected according to the manufacturer’s protocol (Cell Signaling, Charlottesville, VA). Briefly, cells were treated as described in Treatment of Cells, and at the end of the treatment time, cells were washed with PBS and fixed in PBS containing 3% paraformaldehyde for 20 min at 4°C. The cells were then washed three times with Tris-buffered saline-Tween 20 (TBST; 10 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) for 5 min each time and incubated with 1 ml blocking buffer for 45–60 min. The cells were rinsed and incubated in cleaved caspase antibody (1:200 dilution) in TBST/BSA for 24 h at 4°C. They were washed three times, incubated with appropriate fluorescent secondary antibody, and detected using a FITC filter (excitation 490 nm; emission 525 nm).

PI3K Activity

PI3K was estimated according to the manufacturer’s protocol (Echelon, Salt Lake City, UT). Briefly, cells were pretreated with EET, subjected to hypoxia for 8 h, and reoxygenated for 60 min. The cells were then scraped in ice-cold lysis buffer containing (in mM) 137 NaCl, 20 Tris·HCl (pH 7.4), 1 CaCl2, and 1 MgCl2 and 0.1 mM sodium orthovanadate with 1% Nonidet P-40 (NP-40) and 1 mM PMSF. The lysate was centrifuged, and the supernatant was incubated with 5 µl anti-PI3K antibody for 1 h. Protein A agarose beads (60 µl of a 50% slurry) were added, and the lysates were mixed gently for 1 h at 4°C. The immunoprecipitated enzyme was collected by centrifugation for 5 s at 3,000 g and washed once with the lysis buffer and again with buffer of (in mM) 10 Tris·HCl (pH 7.4), 150 NaCl, and 5 EDTA containing 0.1 mM sodium orthovanadate. The phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] substrate was added to the enzyme along with reaction buffer and left at room temperature for 3 h. The kinase reaction was stopped by the addition of 2.5 µl 100 mM EDTA. The plate was then set with standards, blanks, and samples, incubated with the detector protein, and read using a plate reader at 450 nm.

Immunofluorescence Studies for pAkt

Neonatal rat ventricular myocytes were plated on collagen-coated coverslips, pretreated with EET, and exposed to H/R or normoxia for 8 h as described in Detection of Cleaved Caspase-3 Activity. After reoxygenation for 60 min, the cells were fixed in 4% paraformaldehyde, permeabilized in methanol (−20°C), and incubated for 30 min at 37°C with primary monoclonal antibody for pAkt (Ser473; Cell Signaling, Beverly, MA) at a dilution of 1:200 in PBS (0.5×). The samples were washed and incubated with anti-mouse biotinylated secondary antibodies (1:200 dilution; Santa Cruz Biotechnology) for 30 min at 37°C. After being washed with 0.5× PBS, the cells were incubated for 15 min at 37°C with avidin-conjugated 1:500 FITC, followed by 1:1,000 4′,6-diamidino-2-phenylindole (DAPI) (to stain the nuclei) for 5 min at room temperature. The samples were washed again with 0.5× PBS and mounted on microscopic slides. Images were captured using confocal microscopy using appropriate filters for visualization of FITC (described above) and DAPI (excitation, ultraviolet; emission, 461 nm).

Western Blot Analysis

HL-1 cells and neonatal myocytes were cultured in 35-mm dishes to 70% confluency, washed, and incubated with basal medium for 24 h. The cells were treated under normoxia or H/R with or without EETs. They were kept on ice and washed three times with cold PBS. Proteins were solubilized and extracted with 50 µl buffer of 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1× protease inhibitor cocktail (Pharmingen, San Diego, CA), and 1× phosphate inhibitors (Calbiochem, San Diego, CA). The lysates were used to estimate protein content with the Bio-Rad DC Protein Assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein (10–60 µg) from each sample were electrophoresed on a 10% SDS-PAGE with running buffer and transferred to nitrocellulose as described (17, 36). The membranes were treated with primary antibody for XIAP, pBAD, BAD, or pAkt (1:1,000 dilution; Cell Signaling) for 18 h. They were again washed three times before incubating with matched secondary antibody (1:5,000) for 45 min. The protein bands were visualized with enhanced chemiluminescence reagents (ECL or ECL plus; GE Healthcare, Piscataway, NJ).

Functional Evaluation of Contractility of Neonatal Myocytes

Cultured neonatal myocytes (~5 days old) were washed, incubated with basal medium for 8 h, treated with vehicle or EET, and further incubated overnight. The cells were treated under normoxia or exposed to hypoxia for specific times after pretreatment with two
applications of either vehicle or a regioisomer of EET (1 μM of 14,15- or 11,12-EET). In pilot experiments, vehicle-treated cells stopped beating between 30 min and up to 8 h of exposure to anoxic conditions. Test plates from each batch were incubated under hypoxia and observed at half-hour intervals. When ~85% vehicle-treated cells maintained in anoxic conditions stopped beating, all test and control groups were exposed to 1 h of reoxygenation. The dishes were removed from the incubator and immediately observed under a light microscope. Islands of beating cells were counted in a blinded manner in at least four fixed fields/dish from four independent batches of cells. The frequency of beating of at least 25 cells/treatment was also counted in the same samples using a stopwatch. The average number of beating cells/field and the average frequency (beats/min) for each treatment were recorded, decoded, and then used to calculate the mean and SE of the mean for statistical analysis.

Statistical Comparisons

All values are expressed as means ± SE from at least three or more samples in each experiment. Comparisons between controls and treatments were analyzed by ANOVA, followed by Tukey’s test when permitted. Values for \( P < 0.05 \) were considered significant.

RESULTS

EETs Induce Phosphorylation of Akt in Neonatal, Rat, and Cardiac Myocytes

Primary cultures of myocytes were stimulated with increasing concentrations of 14,15-EET to test the effect of the fatty acid on activating the prosurvival kinase Akt (Fig. 1). After 30 min of treatment, the cells were lysed and analyzed by Western blot analysis to determine pAkt levels. As seen in Fig. 1A, there is an increase in pAkt (top) compared with Akt (bottom) in EET-treated cells. Analyses from four independent experiments demonstrated a significant increase in the densitometric ratio of pAkt to Akt versus the vehicle-treated cells at the 1 μM concentration of 14,15-EET (\( n = 4; \) Fig. 1B). At an even higher concentration of 14,15-EET (10 μM), the ratio was lower than for 1 μM of 14,15-EET, as shown in Fig. 1. In cells treated with 10 μM EET, this ratio was statistically indistinguishable to that of the vehicle.

EETs Protect Mouse Atrial (HL-1) and Rat Neonatal Cardiac Myocytes Against H/R-Induced Apoptosis

**MTT cell viability assay.** Both cell types (HL-1 and primary neonatal cardiomyocytes) showed increased survival rates when maintained under normoxia (control) or pretreated with 8,9-, 11,12-, and 14,15-EETs (1 μM of each) compared with the cells that were subjected to H/R after pretreatment with vehicle (Fig. 2). H/R induced a decrease in cell viability to 50% of control as opposed to the cells pretreated with EETs, where the cell survival was ~80% of control.

**Annexin V binding.** We tested all three EET regioisomers, 8,9-, 11,12-, or 14,15-EET (1 μM each), and demonstrated significant protection of HL-1 cells (Fig. 3, A–D) and neonatal myocytes (Fig. 3, E–H) from increased fluorescence due to the binding of annexin V after the induction of apoptosis by H/R. Controls in these experiments were maintained under normoxic conditions. There was a shift in the intensity of fluorescence toward control in EET-pretreated cells compared with cells pretreated with vehicle (labeled H/R; Fig. 3).

**Nuclear fragmentation.** Typical nuclei stained with Hoechst 33342 are shown in Fig. 4. H/R considerably increased DNA condensation in HL-1 cells and myocytes. We observed pyknotic nuclei after H/R in HL-1 cells (Fig. 4A) and neonatal myocytes (Fig. 4B). 14,15-EET protected DNA fragmentation, which is confirmed by the appearance of normal nuclei in EET-pretreated cells.

**Caspase-3 activity.** Caspase-3 activity was increased in HL-1 cells and myocytes (Fig. 5, A and B) after treatment with H/R (reoxygenation was carried out for 2 time points: 8 and 16 h). There was, however, a significant decrease in caspase-3 activity in both cell types that were pretreated with one of the three EET regioisomers (1 μM). We also performed immunofluorescence studies by incubating the treated cells with specific caspase antibody, which only recognizes the activated or cleaved form of the enzyme. The results were similar to the activity assay; H/R-exposed cells showed an increase in fluorescence compared with the H/R group pretreated with EET, both in HL-1 cells (Fig. 6A) and neonatal myocytes (Fig. 6B).

**EETs Activate Multiple Targets of the PI3K/Akt Survival Pathway**

**EETs activate PI3K.** To elucidate a mechanistic pathway of protection by EETs, we evaluated PI3K activity in HL-1 cells (Fig. 7A) and neonatal myocytes (Fig. 7B). There was an increase in PI3K activity in EET-pretreated cells subjected to H/R compared with vehicle-treated cells. The assay was carried out using an immunoprecipitated enzyme to measure the

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**Fig. 2.** EETs increase cell viability after hypoxia and reoxygenation (HR). HL-1 cells (A) and neonatal myocytes (B) were subjected to HR in presence of vehicle or EET. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (see MATERIALS AND METHODS) and normalized to percent control. Bar graphs show means ± SE of percent decrease of viable cells over control (100%). *\( P < 0.01 \) for analyses compared with HR; #\( P < 0.01 \) compared with control.
generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is the product of PI3K from the substrate PIP2, and was measured colorimetrically. The PI3K inhibitor WT blocked the increase in PI3K activity induced by EET.

EETs induce phosphorylation of Akt. Figure 8 depicts the phosphorylation of Akt in H/R-induced HL-1 cells (Fig. 8A) and myocytes (Fig. 8B). The cells treated with 14,15-EET (1 μM) showed an increase in pAkt (green) compared with the H/R-treated cells or normoxic controls (represented graphically in Fig. 8, C and D). Nuclei were counterstained with DAPI (blue). The Akt phosphorylation by each EET regiosomer was further confirmed by the Western blot analysis of proteins (labeled in Fig. 9) from myocytes and HL-1 cells (Fig. 9). Pretreatment with EET increased levels of pAkt (loading controls) compared with vehicle-treated controls. This increase was blocked by WT.

EETs initiate phosphorylation of BAD. To further study the involvement of downstream factors of PI3K/Akt, we performed Western blot analysis for pBAD (Fig. 9) since it is a target for pAkt. We found that there was an increase in the phosphorylation of BAD in EET-pretreated HL-1 cells (right) and neonatal myocytes (left) compared with cells exposed to H/R after pretreatment with vehicle. The expression of BAD (loading control) in the corresponding lysates did not appear altered.

EETs increase intracellular levels of XIAP. XIAP is an antiapoptotic protein that is a downstream effector of the PI3K/Akt prosurvival pathway. We found that there was an increase in XIAP expression in EET-pretreated HL-1 cells (right) and neonatal myocytes (left). These samples were derived from the same protein lysates as BAD (above them in Fig. 9), which demonstrate equal protein loading in each lane.

EETs decrease activity of caspase-9. Caspase-9 is another downstream target of the Akt pathway. Since we found an increase in caspase-3 activity after H/R and its subsequent de-
crease in EET-pretreated cells, we also investigated caspase-9 activity in HL-1 cells and neonatal myocytes (Fig. 10, A and B). We saw a similar trend in caspase-9 activity in these cells with a significant increase in H/R-treated cells and a subsequent decrease when the cells were pretreated with EET.

**EETs Protect Function of Rat Neonatal Cardiac Myocytes Against H/R-Induced Injury**

EETs maintain rhythmic beating of rat neonatal myocytes during H/R. Neonatal rat myocytes beat spontaneously in culture. Exposure to hypoxia retards the rhythmic contractions of these cells (Fig. 11). Beating frequency was preserved by the preincubation with 14,15- or 11,12-EET compared with vehicle. We observed only a few beating cells in each field of myocytes after H/R (the hypoxia was followed by 1 h reoxygenation) compared with those preincubated with 14,15-EETs (1 μM) 16 h before and just before hypoxia (Fig. 11A). The average frequency of these contractions was 108 beats/min under normoxia, which was reduced to 15 beats/min in the few cells still beating after H/R. The cells pretreated with EET were beating at an average frequency of 88 beats/min (Fig. 11B).

![Image of HL-1 cells and neonatal myocytes showing the effects of EET](image-url)

**Fig. 4.** EET blocks fragmentation of nuclei by hypoxia and reoxygenation (H/R). HL-1 cells (A) and neonatal myocytes (B) were subjected to H/R in presence of vehicle or 14,15-EET (1 μM). Nuclei were stained with Hoechst reagent and imaged. There is visible condensation of chromatin (marked with arrows) in serum-deprived cells, which is absent in control cells and reduced in cells pretreated with EET after H/R.

**Fig. 5.** EETs decrease caspase-3 activity. HL-1 cells (A) and neonatal myocytes (B) were subjected to H/R after pretreatment with vehicle (marked HR) or 1 of the 3 regioisomers of EET as labeled. Caspase-3 activity was measured as described in MATERIALS AND METHODS. *P < 0.05, significant difference compared with control (8 h); #P < 0.05, significant difference compared with control (16 h); &P < 0.05, significant difference compared with HR (8 h); $P < 0.05, significant difference compared with HR (16 h). Controls were treated under normoxic conditions. Both cell types showed increased caspase-3 activity after HR, which was attenuated by 1 μM of 8,9-, 11,12-, or 14,15-EET.
DISCUSSION

Our results demonstrate by four independent assays (viability using MTT, nuclear fragmentation by Hoechst staining, increase in the early apoptosis marker annexin V, and activation of the late apoptotic protease caspase-3) that EETs attenuate H/R-induced cell death/apoptosis of cardiomyocytes cultured from the hearts of two rodent species: rats and mice. Importantly, incubation with EETs not only enhanced cell survival but also maintained the contractile function of neonatal myocytes that was attenuated by H/R. Currently, there is no accepted mechanism for the protection of cardiomyocytes by EETs. Our results strongly support the hypothesis that EETs maintain cellular structure and function by the stimulation of the PI3K/Akt cell-survival pathway. We identified five targets that mediate the action of EETs: PI3K, Akt, BAD, XIAP, and caspase-9 (see sche-

Fig. 6. EET decreases intracellular cleaved caspase-3. HL-1 cells (A) and neonatal myocytes (B) were subjected to H/R after pretreatment with vehicle or 14,15-EET (1 μM). After treatment, cells were processed as described in MATERIALS AND METHODS and treated with a caspase-3 antibody, which recognizes only the cleaved caspase and not the procaspase. The cells were then stained with corresponding FITC-tagged secondary antibody and analyzed by fluorescent microscopy. Note increased caspase-3 activity in H/R compared with H/R + 14,15-EET or control (normoxia).

Fig. 7. EETs increase phosphatidylinositol 3-kinase (PI3K) activity. HL-1 cells (A) and neonatal myocytes (B) were subjected to H/R after pretreatment with vehicle or EET in the absence or presence of wortmannin (WT; 0.2–1 μM). Reoxygenation was performed for 1 h. PI3K assays were conducted as mentioned in MATERIALS AND METHODS. The values were determined by plotting the optical density on the standard curve obtained from the known values (standards). *P < 0.001 compared with control or H/R-treated group. PIP$_3$, phosphatidylinositol 3,4,5-trisphosphate.
matic in Fig. 12). Each one of these proteins was affected by EETs in an antiapoptotic manner, defining a mechanism for the observed protection of cardiomyocytes after injury by H/R. EETs may stimulate other pathways in the cell that also have a prosurvival role (e.g., opening of $K_{\text{ATP}}$ channels). Future studies are needed to address whether the activation of PI3K regulates channel function or whether multiple prosurvival pathways must be functional at the same time to protect cardiomyocytes. Understanding clearly how EETs are protective will be a first step toward devel-

Fig. 8. EET increases intracellular pAkt. HL-1 cells (A) and neonatal myocytes (B) were subjected to H/R after pretreatment with vehicle or 14,15-EET. Cells were treated as described in MATERIALS AND METHODS and stained with pAkt antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole, and the cells were viewed under fluorescent microscopy. Images were captured and overlaid as shown. C and D represent the quantitation of green fluorescence, which indicates the increase in pAkt levels. *$P<0.05$ compared with control; #$P<0.05$ compared with H/R.
opposing these fatty acids into therapeutic agents for cardiovascular disease.

The PI3K/Akt pathway is one of the most potent intracellular mechanisms to promote cell survival. For example, in a study of four downstream effectors of growth factor receptors, PI3K, Ras, Raf, and Src, PI3K was the only one to inhibit apoptosis after serum withdrawal (30). Our results indicate that PI3K activity is enhanced by an EET within 30 min of application and also hours later during the first hour of reoxygenation. It is not clear whether EETs directly stimulate PI3K or act indirectly. The best known activators of PI3K include G protein-coupled receptors (GPCRs) (10, 35, 40, 51, 69), receptor tyrosine kinases (23, 33), and glycoprotein 130 (12, 32, 56).

Heterotrimeric G proteins and receptor tyrosine kinases mediate the action of EET. Evidence of cholera toxin-sensitive, high-affinity binding of EETs to mononuclear cells (61, 62) or the activation of $K_{Ca}$ channels via the stimulatory G protein-$G_{s}$ subunit of heterotrimeric G proteins raises the possibility that EETs may stimulate PI3K via GPCR. The sulfonamide derivative of 14,15-EET (20 μM 14,15-EET-SI) induced the association between the EGF receptor and Src-like kinases within 1 min of application at a concentration of 20 μM in renal epithelial cells (7). This high concentration of 14,15-EET-SI stimulated the phosphorylation of the 85-kDa regulatory subunit of PI3K (7). Cross talk with the EGF receptor regulates the angiogenic action of EET in the chick choioallantoic vessels (38). In addition, EETs can transcriptionally upregulate proteins such as the tissue plasminogen activator via $G_{s}$ (45), implying that secondary or late signaling may occur after the synthesis of new proteins or factors that are induced by EET. These observations may explain why a single exposure to free fatty acids, which are rapidly metabolized in cells, may trigger events appearing many hours later.

One downstream target of PI3K is the kinase Akt (67), which is reported to be activated during the intracellular signal transduction of many receptors and survival factors. Members of this family include Akt1, Akt2, and Akt3 (42). Akt, also known as protein kinase B or RAC-PK (related to A and C kinases), is a serine-threonine kinase. PI3K phosphorylates inositol lipids that recruit and modify several targets containing...
Pleckstrin homology (PH) domains including Akt and 3-phosphoinositide-dependent kinase-1 (PDK-1) (4, 22, 31). Akt1 is activated by phosphorylation first on Thr<sup>308</sup> by PDK-1 and subsequently on Ser<sup>473</sup> (53). PDK-1 has a PH domain and is, therefore, also recruited to the membrane after the activation of PI3K. A number of downstream targets that regulate apoptosis are modulated by pAkt including members of the Bcl-2/CED9 family Bax, Bak, and BAD (14, 26, 63, 68), XIAP, caspase-9, GSK-3β, transcription factors of the forkhead, NF-κB families, and endothelial nitric oxide synthase (18, 34, 49, 59). BAD and caspase-9 are inactivated after phosphorylation by Akt (1), whereas XIAP is stabilized by it. Therefore, Akt promotes cell survival by ultimately modifying the core death machinery.

Akt primarily triggers the phosphorylation of BAD at Ser<sup>136</sup>, which is sufficient to promote survival. The phosphorylation of BAD leads to the prevention of cell death via a mechanism that involves the selective association of pBAD with isoforms of the scaffold protein 14–3–3. This interaction induces the release of antiapoptotic-binding partners of BAD such as Bcl-x<sub>L</sub> or Bcl-2 (14, 24, 65). 14–3–3 isoforms associate with a number of cellular signaling molecules including the kinase suppressor of Ras, cdc25, Raf-1, and PI3K (3, 39). Growth factor deprivation or apoptotic signals cause a removal of the phosphates, which allow BAD to bind to antiapoptotic Bcl-2, ultimately resulting in the displacement of voltage-dependent anion channel-2 of the mitochondria, increasing mitochondrial outer membrane permeabilization and inducing apoptosis. BAD is, therefore, a proapoptotic molecule when it is not phosphorylated.

XIAP is, as its name suggests, an inhibitor of apoptosis that belongs to the inhibitor of apoptosis family of proteins with an evolutionarily conserved role in regulating programmed cell death (15, 16). It binds to and blocks caspases-9, -3, and -7 (16). The protein contains baculovirus inhibitors of apoptosis repeats, which limit substrate access to catalytic sites of activated caspases. In addition, this protein prevents the dimerization of procaspase-9 (57) and protects the cells from accidental activation of caspases. XIAP is an important antiapoptotic protein since it attenuates the major inhibitor of the extrinsic pathway, caspase-9, as well as the common mediator of both the intrinsic as well as extrinsic pathways, caspase-3 (13). Our results (Fig. 9) demonstrate reduced levels of this protein after H/R in both types of cultured myocytes. Pretreatment with EETs impressively reduces this fall in the level of XIAP induced by H/R.

Although this is the first report describing antiapoptotic actions of EETs on cardiomyocytes, the enhanced survival of other cultured cells by these fatty acids and also by epoxygenase overexpression has been reported. Examples of cell types so affected include porcine kidney proximal tubule-like epithelial cell line (LLCPK14) (6), primary human coronary and pulmonary microvascular endothelial cells (17), bovine aortic endothelial cells (66), and the human carcinoma cell line Tca-8,113 (29). The PI3K/Akt pathway has been implicated in all of these studies, although much higher concentrations of 14,15-EET (10–20 μM) were used in the kidney cells (6), where Akt kinase activity was increased 10 min after the addition of 14,15-EET to the LLCPK14s. Levels of pAkt were higher after at least 12 h of treatment of the carcinoma cells with 8,9-, 11,12-, or 14,15-EET (100 nM), followed by the

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**Fig. 11.** EET protects contractility of primary neonatal myocytes in culture. Neonatal myocytes were subjected to H/R after pretreatment with vehicle or 14,15-EET (1 μM). The number of beating cells in a microscope field (A) and the frequency (beats/min; B) were counted as described in MATERIALS AND METHODS (a total of 3–5 batches of cells were examined for each treatment). Pretreatment with EET increased both total number and frequency of beating cells compared with myocytes that were exposed to H/R in the presence of vehicle. *P < 0.05, significant difference compared with control; #P < 0.05, significant difference compared with H/R.

**Fig. 12.** Schematic representation of a pathway for protection of cardiomyocytes by EET via the PI3K/Akt prosurvival pathway. We have reported that pretreatment with EET increases 1) PI3K activity, 2) phosphorylation of Akt, 3) phosphorylation of BAD, 4) intracellular XIAP, and 5) inhibition of activity of caspases-9. Each of these events decreases activity of caspase-3, a late effector, to protect the cardiomyocyte from apoptosis by H/R. These targets are known to align in a signaling cascade as illustrated in the schematic. It should be noted that the results do not implicate absence of other prosurvival or mitotic pathways that may also be targeted by EETs. RTK, receptor tyrosine kinases.
induction of apoptosis by tumor necrosis factor-α for 12 h (29). Interestingly, the expression of the 110-kDa PI3K subunit was increased after the overexpression of epoxigenase enzymes that catalyze formation of EET in bovine aortic endothelial cells (66).

Hence, we demonstrate that EETs prevent apoptosis in rat neonatal cardiac myocytes and a mouse atrial cardiomyocyte cell line (HL-1) subjected to H/R injury. We report for the first time that treatment with EET enhances PI3K activity (Fig. 7). This treatment also attenuates both the increase in activity of caspase-9 and the fall in the levels of intracellular XIAP induced by H/R. We have confirmed that EETs increase phosphorylation of Akt that is upstream of these effectors. Together, these observations support mechanistic evidence for the protective effect of EETs in cardiomyocytes to prevent H/R-induced cell death. These data make it essential to investigate whether the activation of potassium channels (especially KATP channels), the mechanism favored by other investigators for the protection of myocardium by EETs, is independent of PI3K or occurs by cross talk in parallel to this prosurvival pathway.

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REFERENCES

PROTECTION OF CARDIOMYOCYTES BY EETs VIA PI3K/Akt


