Journal of Molecular and Cellular Cardiology 53 (2012) 43–52

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Role of PI3Kα and sarcolemmal ATP-sensitive potassium channels in epoxyeicosatrienoic acid mediated cardioprotection

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ARTICLE INFO

Article history:
Received 8 November 2011
Received in revised form 9 April 2012
Accepted 16 April 2012
Available online 27 April 2012

Keywords:
EET
pmKATP channel
PI3K and Ischemia-Reperfusion

ABSTRACT

Aims: Epoxyeicosatrienoic acids (EETs) are cytochrome P450 epoxygenase metabolites of arachidonic acid that have known cardioprotective properties. While the mechanism(s) remains unknown, evidence suggests that phosphoinositide 3-kinase (PI3K) and sarcolemmal ATP-sensitive potassium channels (pmK_{ATP}) are important. However the role of specific PI3K isoforms and corresponding intracellular mechanisms remains unknown.

Methods and results: To study this, mice hearts were perfused in Langendorff mode for 40 min of baseline and subjected to 20 or 30 min of global no-flow ischemia followed by 40 min of reperfusion. C57BL6 mice perfused with 11,12-EET (1 μM) had improved postischemic recovery, whereas co-perfusion with PI3Kα inhibitor, PI-103 (0.1 μM), abolished the EET-mediated effect. In contrast, blocking of PI3Kβ or PI3Kγ isoforms failed to inhibit EET-mediated cardioprotection. In addition to the improved post-ischemic recovery, increased levels of p-Akt, decreased calcineurin activity and decreased translocation of proapoptotic protein BAD to mitochondria were noted in EET-treated hearts. Perfusion of 11,12-EET to Kir6.2 deficient mice (pmK_{ATP}) failed to improve postischemic recovery, decrease calcineurin activity and translocation of proapoptotic protein BAD, however increased levels of p-Akt were still observed. Patch-clamp experiments demonstrated that 11,12-EET could not activate pmK_{ATP} currents in myocytes pre-treated with PI-103. Mechanistic studies in H9c2 cells demonstrate that 11,12-EET limits anoxia–reoxygenation triggered Ca^{2+} accumulation and maintains mitochondrial $\Delta\Psi m$ compared to controls. Both PI-103 and glibenclamide (10 μM, pmK_{ATP} inhibitor) abolished EET cytoprotection.

Conclusion: Together our data suggest that EET-mediated cardioprotection involves activation of PI3Kα, upstream of pmK_{ATP}, which prevents Ca^{2+} overload and maintains mitochondrial function.

1. Introduction

In recent years, it has become evident that metabolites of polyunsaturated fatty acids act as critical intracellular mediators, maintaining cardiac homeostasis and initiating protective responses to cellular stress. Arachidonic acid (AA) is a polyunsaturated fatty acid normally found esterified to cell membranes that can be released in response to several stimuli including ischemia [1]. Free AA is further metabolized by cyclooxygenases, lipoxygenases, and cytochrome P450 (CYP) epoxygenases to numerous products collectively termed eicosanoids with differing cellular function in health and disease [2]. CYP epoxygenases metabolize AA to four regioisomer (5,6-, 8,9-, 11,12- and 14,15) products, epoxyeicosatrienoic acids (EETs) [2]. Elevated levels of EETs are known to render cardiac and extra-cardiac protection against injury [3–5]. While the exact mechanism remains unknown, evidence indicates EETs protect the heart by modulating ion channels such as pmK_{ATP} and activating PI3K pathways, thereby effecting cardiovascular physiology and function [5–8].

Cardiac pmK_{ATP} channels are involved in regulating ionic homeostasis under conditions of metabolic stress and have demonstrated cardioprotective effects towards ischemia reperfusion injury [9,10]. pmK_{ATP} channels can be activated during cardiac ischemia when cytoplasmic ATP is depleted and affects membrane excitability. Activation of pmK_{ATP} channels during ischemia leads to shortening of the cardiac action potential and opposes membrane depolarization [11] consequently reducing intracellular calcium overload thus limiting myocardial damage [12]. Recent data from animal models of IR injury have demonstrated that EET-mediated activation of pmK_{ATP} channels

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doi:10.1016/j.yjmcc.2012.04.008

improves cardiac functional recovery [3,5,7,8]. While EETs have been shown to be activators of pmK_{ATP} by reducing channel sensitivity to ATP under normoxic conditions [8], the mechanism(s) of how this occurs during IR injury remains enigmatic.

PI3Ks are members of a family of lipid kinases that regulate a range of cell survival, growth and metabolic processes [13]. Based on amino acid sequence, structure and mode of activation, these are divided into three classes (I, II and III) [14]. Class I PI3K catalyzes the addition of a phosphate group to the 3′ position of the phosphatidylinositol (PIP) [13,14], activating other intracellular signaling kinases [13,14] and ion channels [15] with distinct roles in regulating cardiac function [16–18]. Enhanced activation of class 1 PI3Ks and signaling pathways involving downstream kinases, such as Akt and GSK-3β, during IR injury results in reduced cell death and infarct size [14]. PI3K pathways have been demonstrated to be involved in EET cardioprotective signaling [5,6].

While EET-mediated cardioprotection involves PI3K and pmK_{ATP} channel dependent events, the specific intracellular signaling mechanism(s) remains unresolved. In this study, we identify the specific PI3K isoform (PI3Kα) involved in the EET protective mechanism and investigate the link between PI3K and pmK_{ATP} channels in EET cardioprotection. Furthermore, our results suggest that activation of PI3Kα and pmK_{ATP} channels by EETs renders protection by decreasing Ca^{2+} overload following ischemic injury, limiting mitochondrial damage.

2. Materials and methods

For an expanded Material and methods section, see the on-line data supplement.

2.1. Animals and isolated heart perfusion

Commercially available C57BL/6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ). The PI3Kγ−/− [19] and Kir6.2−/− [10] (K_{ATP} channel-deficient) mice were used as previously described. All studies were carried out using 2–3 month old mice weighing 25–30 g. Mice were euthanized intraperitoneally with sodium pentobarbital (Euthanyl, 500 mg/kg) and following non-responsiveness to external stimulation hearts were isolated and perfused in the Langendorff mode. Hearts were subjected to 20/30 min no-flow global ischemia, followed by 40 min of reperfusion (Supplementary Fig. 1A) [20]. Hearts were perfused with 11,12-EET or 14,15-EET and/or PI-103 (PI3Kα inhibitor, Cayman Chemicals, USA), TGX-221 (PI3Kβ inhibitor, Cayman Chemicals, USA), wortmannin (pan-specific inhibitor, Sigma-Aldrich, Canada) or 5-hydroxydecanoate (5-HD, mitoK_{ATP} channel inhibitor, Sigma-Aldrich, Canada). In all the experiments, the drugs were added 5 min before ischemia and were present in the heart throughout the reperfusion period. Experiments were conducted according to strict guidelines provided by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS).

2.2. Electrophysiology

Whole-cell K_{ATP} channel currents were recorded from freshly isolated adult mouse ventricular myocytes that were placed in a recording chamber on the stage of an inverted microscope and superfused with an extracellular bath solution containing NaCl 20 mM, KCl 4.5 mM, choline chloride 130 mM, $CaCl_2$ 1 mM, $CoCl_2$ 2 mM, $MgCl_2$ 2 mM, HEPEs 10 mM, and glucose 5.5 mM, pH 7.4. After gigaohm seal formation, K_{ATP} channel currents were elicited by rupturing the patch and dialyzing the cells with a pipette solution containing (mM): KCl 140, $CaCl_2$ 0.465 (200 nM free Ca^{2+}), $MgCl_2$ 0.5, Na_2ATP 1, Na_2GTP 0.5, HEPES 1, EGTA 1, pH 7.3. Currents were filtered at 2 kHz and digitized at 5 kHz using an Axopatch 200B amplifier and pClamp 8.2 software (Axon Instruments, Union City, CA, USA). Experiments were performed at a negative holding potential of –100 mV at room temperature (21–23 °C).

2.3. Cell culture experiments

H9c2 cells (American Type Culture Collections, Manassas, VA) were cultured in 75 cm^2 flasks at 37 °C in an atmosphere of 5% CO_2/95% air. DMEM media with phenol red, supplemented with 10% bovine serum albumin, and antibiotics such as penicillin, streptomycin and amikacin, were used to nourish the cells but in some experiments we used Ca^{2+} free DMEM media. In all experiments, cells were seeded on petri dishes and treated with either 11,12-EET, PI-103 or glibenclamide (GLIB) (Supplementary Fig. 1B). Cell viability was assessed using trypan blue dye exclusion assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) assay. Reactive oxygen species (ROS) levels were measured by using 2.5 μM DCF dye (Invitrogen, USA) in Hank's buffer following anoxia–reoxygenation. Mitochondrial membrane potential was studied following oxidative stress using tetramethylrhodamine ethyl ester (TMRE) (Invitrogen, USA) under an epiflourescence microscope. Ca^{2+} overloading experiments were conducted under oxidative stress using Fluo-4(2 μM) under an epiflourescence microscope.

2.4. Calcineurin and caspase-3 assay

The cytosolic fraction was separated from the heart tissue as described [4,7] and calcineruin enzyme activity assay was carried out as per the kit instructions (Calbiochem, Colorimetric based, Calcineurin assay kit). Caspase-3 activity was assessed in heart cytosolic fractions using a spectrofluorometric assay as described [20].

Fig. 1. Identification of the PI3K isoform and the role of pmK_{ATP} channels in EET-mediated cardioprotective mechanism. A, Histogram of the functional recovery at 40 min reperfusion expressed as percentage of baseline LVDP from C57BL6 hearts treated with vehicle, 11,12-EET (1 μM) or Wortmannin (200 nM) following 30 min ischemia. Values represent mean ± SEM, $n = 3$–6 per group; *, $P<0.05$ vs. vehicle control; †, $P<0.05$ vs. 11,12-EET treated group. B, Histogram of the percentage of LVDP change at 40 min reperfusion compared to baseline from the hearts perfused with vehicle, 11,12-EET (1 μM) or PI-103 (0.01 μM, 0.1 μM or 1 μM) following 30 min of ischemia. Values represent mean ± SEM, $n = 5$–6 per group; *, $P<0.05$ vs. vehicle control; †, $P<0.05$ vs. vehicle treated group. C, Histogram of the percentage of LVDP change at 40 min reperfusion compared to baseline from the hearts perfused with vehicle, 11,12-EET (1 μM) or TGX-221(0.01 μM) following 30 min of ischemia. Values represent mean ± SEM, $n = 3$–6 per group; *, $P<0.05$ vs. vehicle control; †, $P<0.05$ vs. 11,12-EET (1 μM) treated group; ‡, $P<0.05$ vs. control group treated with TGX-221. D, Histogram of the percentage of LVDP change at 40 min reperfusion compared to baseline from the PI3Kγ−/− hearts perfused with vehicle, 11,12-EET (1 μM) or PI-103 (1 μM) following 30 min of ischemia. Values represent mean ± SEM, $n = 3$–6 per group; *, $P<0.05$ vs. vehicle control; †, $P<0.05$ vs. 11,12-EET (1 μM) treated group. E, Histogram of the percentage of LVDP change at 40 min reperfusion compared to baseline from the PI3Kγ−/− hearts perfused with 11,12-EET (1 μM) or TGX-221 (0.01 μM) following 30 min of ischemia. Values represent means ± SEM, $n = 3$–6 per group; †, $P<0.05$ vs. control group treated with TGX-221. F, Histogram of the functional recovery at 40 min reperfusion expressed as a percentage of baseline LVDP from WT and Kir6.2−/− hearts perfused with vehicle or 11,12-EET (1 μM) following 20 min ischemia. Values represent mean ± SEM, $n = 6$–11 per group; *, $P<0.05$ vs. vehicle control; †, $P<0.05$ vs. 11,12-EET treated group. G, Histogram of the functional recovery at 40 min reperfusion expressed as a percentage of baseline LVDP from the hearts perfused with vehicle, 11,12-EET (1 μM) or 5-HD (100 μM) following 30 min of ischemia. Values represent mean ± SEM, $n = 3$–6 per group; *, $P<0.05$ vs. vehicle control; †, $P<0.05$ vs. 11,12-EET treated group.