British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01093.x
www.brjpharmacol.org

RESEARCH PAPER

Cardioprotective effect of a dual acting epoxyeicosatrienoic acid analogue towards ischaemia reperfusion injury

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Keywords
ischaemia-reperfusion; cardioprotection; EET surrogates; mitochondria and sEHi

Received
23 June 2010
Revised
23 September 2010
Accepted
9 October 2010

BACKGROUND AND PURPOSE

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 epoxygenase metabolites of arachidonic acid that are metabolized into dihydroxyepoxyeicosatrienoic acids (DHET) by soluble epoxide hydrolase (sEH). The current investigations were performed to examine the cardioprotective effects of UA-8 (13-(3-propylureido)tridec-8-enoic acid), a synthetic compound that possesses both EET-mimetic and sEH inhibitory properties, against ischaemia-reperfusion injury.

EXPERIMENTAL APPROACH

Hearts from C57BL/6 mice were perfused in Langendorff mode and subjected to ischaemia reperfusion. Mechanistic studies involved co-perfusing hearts with either 14,15-EEZE (a putative EET receptor antagonist), wortmannin or PI-103 (class-I PI3K inhibitor). H9c2 cells were utilized to investigate the protective effects against mitochondrial injury following anoxia reoxygenation.

KEY RESULTS

Perfusion of UA-8 significantly improved postischaemic left ventricular developed pressure (LVDP) and reduced infarction following ischaemia reperfusion compared with control and 11,12-EET. UA-7 (13-(2-(butylamino)-2-oxoacetamido)tridec-8(Z)-enoic acid), a compound lacking sEH inhibitory properties, also improved postischaemic LVDP, while co-perfusion with 14,15-EEZE, wortmannin or PI-103 attenuated the improved recovery. UA-8 prevented anoxia-reoxygenation induced loss of mitochondrial membrane potential and cell death in H9c2 cells, which was blocked by co-treatment of PI-103.

CONCLUSIONS AND IMPLICATIONS

UA-8 provides significant cardioprotection against ischaemia reperfusion injury. The effects are attributed to EETs mimetic properties, which limits mitochondrial dysfunction via class-I PI3K signalling.

Abbreviations

AA, arachidonic acid; DHET, dihydroxyepoxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; IR, ischaemia-reperfusion; K_{ATP}, ATP-sensitive potassium channels; LVDP, left ventricular developed pressure; PI3K, phosphoinositide 3-kinase; sEH, soluble epoxide hydrolase; *t*AUCB, *trans*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid; TMRE, tetramethylrhodamine ethyl ester; UA-7, 13-(2-(butylamino)-2-oxoacetamido)tridec-8(Z)-enoic acid; UA-8, 13-(3-propylureido)tridec-8-enoic acid; $\Delta\Psi_m$, mitochondrial membrane potential

Introduction

Arachidonic acid (AA) metabolites generated by cytochrome P450 epoxygenases (CYP), epoxyeicosatrienoic acids (EETs), are important lipid mediators involved in regulating cardiac function and protection against ischaemia reperfusion injury. EETs exist as four regioisomers (5, 6-; 8, 9-; 11, 12- and 14, 15-EET) that are rapidly converted to the corresponding and less biologically active dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) (Kim *et al.*, 2004; Morisseau and Hammock, 2005). EET-mediated actions can be reduced by metabolism via beta-oxidation, esterification and autooxidation (Spearman *et al.*, 1985; Fang *et al.*, 2002; Seubert *et al.*, 2006; Chiamvimonvat *et al.*, 2007). Elevation of intracellular EETs has been shown to have cardioprotective effects against ischaemia-reperfusion (IR) injury. Although the precise mechanism(s) remains elusive, studies have demonstrated that the protective mechanism(s) involves modulation of ion channels like ATP-sensitive potassium channels (K_{ATP}) and signalling through phosphoinositide 3-kinase (PI3K) pathways targeting the mitochondria (Seubert *et al.*, 2004; 2006; Gross *et al.*, 2007; Dhanasekaran *et al.*, 2008; Katragadda *et al.*, 2009).

EETs are chemically and metabolically labile; a rapid metabolism, low solubility and storage issues limit their use as therapeutic agents and pharmacological tools (Kaspera and Totah, 2009). Indeed, difficulty arises with long-term treatment using EETs because they are rapidly metabolized or incorporated into membranes (Spector *et al.*, 2004). As such, considerable interest has arisen in developing methods to enhance the bioavailability of EETs. To overcome these limitations, the administration of pharmacological inhibitors of CYP epoxygenases (MS-PPOH) (Seubert *et al.*, 2004) and sEH [*trans*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*AUCB)] (Chaudhary *et al.*, 2009a) have been utilized. However, current sEH inhibitors are limited in effect as they rely on endogenous EET production, which undergoes further metabolism and/or incorporation into membranes.

Recently developed EET-analogues possessing EET-mimetic and sEH inhibitory properties contain several key features: (i) a partially saturated carbon backbone to avoid autooxidation and improve physical stability; (ii) a *cis*-$\Delta^{8,9}$-olefin for EET-mimetic activity; and (iii) 1,3-disubstituted urea for sEH inhibitory properties that prolongs the half-life (Figure 1A) (Falck *et al.*, 2009). In the present study, we report cardioprotective effects of the novel EET analogue, UA-8. Moreover, our data demonstrate marked reduction in infarction and reduced mitochondrial damage at nanomolar concentrations that involves Class-I PI3K-dependent pathway.

Methods

Animals

Male and female mice aged 3–5 months, weighing 22–33 g were used in all experiments and were treated in accordance with the guidelines of Health Science Laboratory Animal Services, University of Alberta. C57BL/6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ). A colony of mice with targeted disruption of the Ephx2 gene (sEH null) and backcrossed onto a C57BL/6 genetic background for more than seven generations, is maintained at the University of Alberta.

Perfusion of isolated hearts

Hearts were perfused in the Langendorff mode as described previously (Batchu *et al.*, 2009; Chaudhary *et al.*, 2009b). Briefly, hearts from mice were perfused in a retrograde fashion at constant pressure (90 cmH_2O) with continuously aerated (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C. Hearts were perfused for 40 min (stabilization), and then subjected to 20 min of global no-flow ischaemia, followed by 40 min reperfusion. Recovery of contractile function was taken as left ventricular developed pressure (LVDP) at 40 min reperfusion and expressed as a percentage of pre-ischaemic LVDP. For some experiments, hearts were perfused with 11,12-EET (1 μM), UA-8 (13-(3-propylureido)tridec-8-enoic acid; 0.01–1 μM), UA-7 (13-(2-(butylamino)-2-oxoacetamido)tridec-8(Z)-enoic acid; 1 μM), 14,15-EEZE (10 μM), MS-PPOH (50 μM), wortmannin (1 μM, Sigma-Aldrich, Canada) and PI-103 (1 μM, Cayman Chemicals, USA) (Figure 1A). In all the experiments, hearts were stabilized for 40 min and then subjected to 20 min of ischaemia followed by 40 min of reperfusion (Figure 1B). To determine the amount of infarction, after 2 h reperfusion following 20 min ischaemia, hearts were incubated with a 1% solution of 2,3,5-triphenyltetrazolium chloride dissolved in Krebs-Henseleit buffer at 37°C for 10 min, then fixed in formalin and cut into thin cross-sectional slices. The area of infarction was quantified by measuring stained (red, live tissue) and unstained (white, necrotic) regions and compared with total area of the slice.

Immunoblotting

Crude cytosol fractions were prepared from frozen mouse hearts as previously described (Batchu *et al.*, 2009; Chaudhary *et al.*, 2009b). Protein was resolved on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies to phospho- and total-AKT or phospho- and total-GSK3β (1:1000, Cell Signalling Technology, Inc., USA). Relative band intensities were expressed in arbitrary units assessed using Image J software (NIH, USA).

Cell culture experiments

H9c2 cells (ATTC, Manassas, VA) were cultured under anoxic conditions (37°C in an atmosphere of 5% CO_2/95% air). DMEM media with phenol red was supplemented with 10% foetal bovine serum, and antibiotics penicillin, streptomycin and amikacin. In all the experiments cells were treated with 11, 12-EET (1 μM) or UA-8 (0.1 μM). Anoxic conditions were generated in an air tight chamber (MIC101, Billups-Rothenberg, Inc.) and maintained for 6 h followed by 16 h of re-oxygenation (Figure 1B).

Cell viability was assessed by using trypan blue dye exclusion assay. Following anoxia – re-oxygenation cells were collected and centrifuged. The supernatant was discarded and the resultant pellet was resuspended in homogenization buffer as previously described (Seubert *et al.*, 2006;

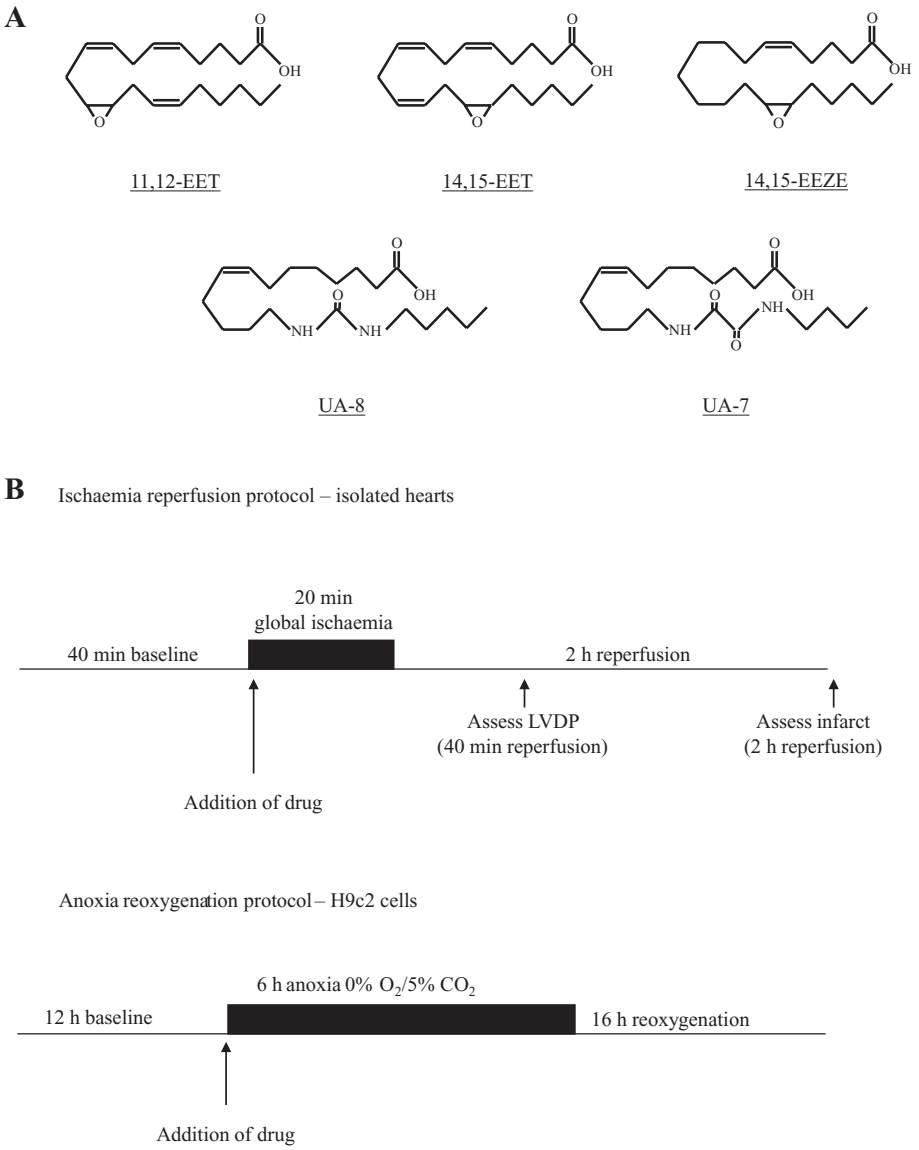

Figure 1

(A) Structures of 11,12-EET, 14,15-EET, 14,15-EEZE, UA-7 and UA-8. (B) Experimental protocols for ischaemia reperfusion and anoxia reoxygenation. LVDP, left ventricular developed pressure.

Dhanasekaran *et al.*, 2008; Chaudhary *et al.*, 2009b). A 50 µL aliquot was mixed with 0.4% trypan blue dye (Sigma-Aldrich, Oakville, ON, Canada) in a 1:1 ratio and allowed to incubate for 3 min. This mixture was then placed on the haemocytometer and viable (unstained) and dead (blue-stained) cells were counted.

Caspase-3 activity was assessed using a spectrofluorometric assay as described previously (Zhang *et al.*, 2009). Briefly, caspase-3 activity was determined in cytosolic fractions by monitoring the release of 7-amino-4-methylcoumarin (AMC) by proteolytic cleavage of the peptide Ac-DEVD-AMC (20 µM) (Sigma-Aldrich, Oakville, ON, Canada). Fluorescence was monitored at wavelengths of 380 nm (excitation) and 460 nm (emission). Specific activities were determined to be within the linear range of a standard curve established with AMC.

Mitochondrial membrane potential ($\Delta\psi_m$)

The $\Delta\Psi_m$ was studied by loading H9c2 cells with 150 nM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen, USA) to record changes in $\Delta\Psi_m$. TMRE is a cationic dye attracted to polarized mitochondria membranes, a reflection of mitochondrial function. Cells were incubated with 150 nM TMRE for 20 min after 6 h anoxia and 16 h re-oxygenation. Changes in fluorescence were recorded over a 10-min period at 37°C using a Zeiss Axio Observer Z1 inverted epifluorescence microscope to take z-stack images for two channels

every 10 s with a 500 ms exposure time. Cells were observed under a PlanApo 40× oil immersion objective lens (Zeiss) with a numerical aperature value of 1.4. TMRE was excited at 555 nm and emission was recorded with a band-pass filter of 575–640 nm. Measurements were taken from individual experiments and intensities were quantified relative to background levels.

Statistical analysis

Values are expressed as the mean ± SEM. Statistical significances between groups were determined by the use of Student's *t*-test and one-way ANOVA followed by Duncan's tests to assess multiple group comparisons. Values were considered significant if $P < 0.05$.

Results

Cardioprotective effects of UA-8

To demonstrate whether UA-8, a structural analog of EET with sEH inhibition properties (Figure 1A), had similar cardioprotective effects as EETs, we first performed a dose–response study. We perfused C57BL/6 mouse hearts with 0, 0.01, 0.1, 0.5 and 1 μM of UA-8 and monitored LVDP for postischaemic functional recovery (Table 1). Hearts perfused with UA-8 had significantly improved postischaemic recovery of LVDP compared with control mice (Figure 2A). The improved postischaemic recovery followed a rapid dose–response with the improved functional recovery occurring at low concentrations (0.1 μM 80.0 ± 4.0%) compared with vehicle-treated hearts (34.0 ± 4.0%). The improved postischaemic functional recovery from UA-8 (0.1 μM) was significantly higher compared with functional recovery from sEH null mice (49.0 ± 5.0%) or hearts perfused with 11,12-EET (1 μM) (60.0 ± 7.0) (Figure 2B). Infarct size was assessed after 20 min of global ischaemia and following 2 h reperfusion with UA-8 (0.1 μM) – injury was measured as percentage of the infarction region to the area at risk (IS/AAR). A significant decrease in infarct size was observed in the hearts treated with UA-8 compared with both vehicle-treated and EET-treated hearts (Figure 3C). Together these data suggest that UA-8 is a potent and stable agent and improves postischaemic functional recovery similar to that of natural EETs at nanomolar concentrations.

Protective effects of UA-8 are due to structural similarity to EET

To determine whether the improved ventricular recovery induced by UA-8 (0.1 μM) was due to EET structural properties or inhibition of sEH activity, we perfused hearts with UA-7, a synthetic compound that is structurally similar to UA-8 but possesses no sEH inhibition properties (Figure 1A). Hearts perfused with UA-7 (1 μM) had significantly improved post ischaemic recovery of LVDP compared with control mice, similar to the improved recovery seen with UA-8 (UA-7 70.0 ± 8.0%; UA-8 71.0 ± 11.0%; vehicle 34.0 ± 4.0%). Next, hearts were co-perfused with the putative pan-EET antagonist 14,15-EEZE and either UA-8 or UA-7. Interestingly perfusion of this antagonist completely abolished the improved postischaemic functional recovery induced by both UA-8 and

Table 1
Cardiac parameters for UA-8 dose response

	Vehicle control (n = 11)	UA-8 (0.01 μM) (n = 4)	UA-8 (0.1 μM) (n = 6)	UA-8 (0.5 μM) (n = 4)	UA-8 (1 μM) (n = 5)
Isolated perfused heart – preischaemic					
LVDP (cmH_2O)	114.0 ± 7.0	122.0 ± 11.0	100.0 ± 4.0	122.0 ± 2.0	108.0 ± 7.6
Rate of contraction, dP/dt_{max} ($cmH_2O \cdot ms^{-1}$)	3856 ± 485	4235 ± 258	3628 ± 371	5428 ± 1002	3056 ± 477
Rate of relaxation, $-dP/dt_{min}$ ($cmH_2O \cdot ms^{-1}$)	−2885 ± 370	−3322 ± 237	−3244 ± 291	−4527 ± 737	−2613 ± 413
HR, perfused ($beats \cdot min^{-1}$)	330 ± 21	281 ± 57	328 ± 23	318 ± 16	289 ± 34
Isolated perfused heart – postischaemic (R40)					
LVDP (mmHg)	39.0 ± 4	58.0 ± 5	80.0 ± 7*	85 ± 16*	77 ± 13*
Rate of contraction, dP/dt_{max} ($cmH_2O \cdot ms^{-1}$)	1326 ± 209	1600 ± 147	2588 ± 404*†	2963 ± 506*†	2336 ± 344*†
Rate of relaxation, $-dP/dt_{min}$ ($cmH_2O \cdot ms^{-1}$)	−1108 ± 179	−1737 ± 282	−2785 ± 347*†	−2712 ± 455*	−2273 ± 375*†
HR, perfused ($beats \cdot min^{-1}$)	303 ± 18	279 ± 18	336 ± 16	297 ± 12	237 ± 40

Hemodynamic parameters were measured in isolated-perfused hearts.
Values represent mean ± SEM, *$P < 0.05$ versus vehicle control, †$P < 0.05$ versus UA-8 (0.01 μM).
HR, heart rate; LVDP, left ventricular developed pressure.

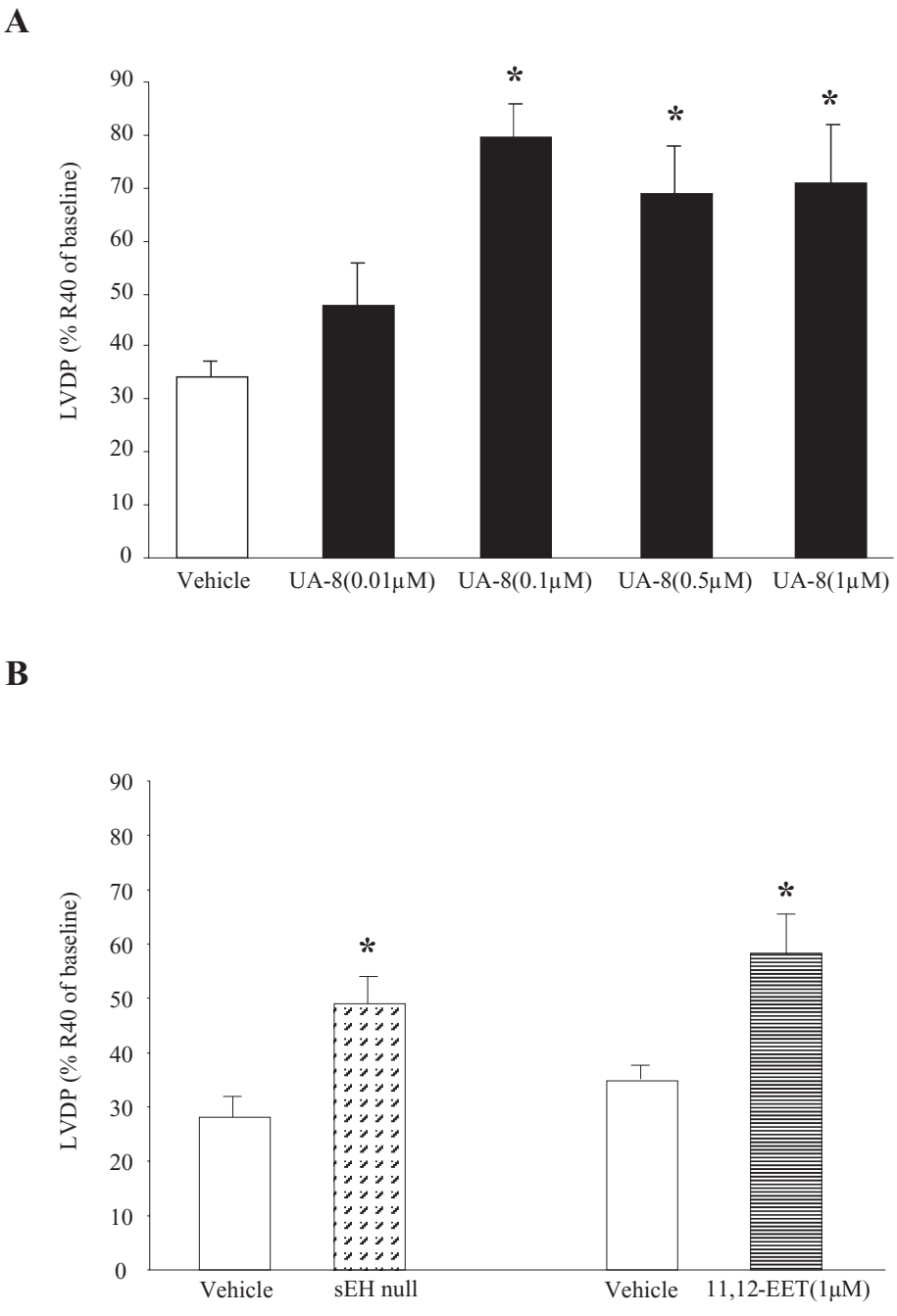

Figure 2

Cardioprotective effect of UA-8. (A) Histogram of the postischaemic functional recovery of LVDP at 40 min of reperfusion, expressed as percentage of baseline LVDP from C57BL/6 hearts treated with vehicle or UA-8 (0.01, 0.1, 0.5 and 1 μM). Values represent means ± SEM, $n = 5–11$ per group; *$P < 0.05$ versus vehicle control. (B) Histogram of the postischaemic functional recovery of LVDP at 40 min reperfusion expressed as percentage of baseline LVDP from sEH null and C57BL/6 hearts treated with vehicle or 11,12-EET (1 μM). Values represent means ± SEM, $n = 7–11$ per group; *$P < 0.05$ versus vehicle control. LVDP, left ventricular developed pressure; sEH, soluble epoxide hydrolase.

UA-7 (Figure 3A,B). Finally, hearts were co-perfused with UA-8 and the CYP epoxygenase inhibitor MS-PPOH (50 μM) (Figure 3A), to block the endogenous EET production. However, the administration of this inhibitor did not have any effect on the postischaemic recovery induce by UA-8. Taken together, these data suggest that UA-8-mediated protection results from its ability to mimic the structure of EET rather than inhibition of sEH activity.

PI3K-mediated effects of UA-8

To further investigate the role of PI3K in UA-8-mediated protection, we assessed the expression levels of phosphorylated

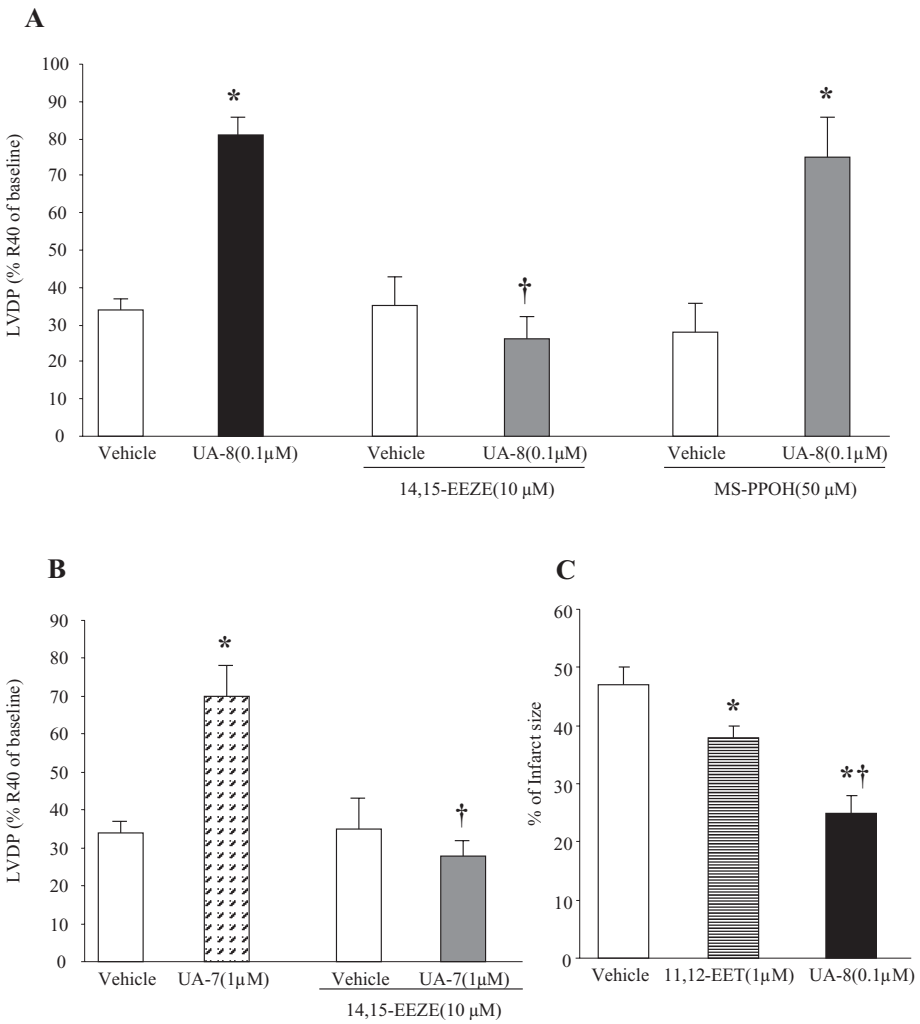

Figure 3

Effect of UA-8 on postischaemic contractile function and infarct size. (A) Histogram of the functional recovery at 40 min reperfusion expressed as percentage of baseline LVDP from C57BL/6 hearts treated with vehicle or UA-8 (0.1 μM), 14,15-EEZE (10 μM) or MS-PPOH (50 μM) following 20 min of ischaemia. Values represent mean ± SEM, n = 3–11 per group; *P < 0.05 versus vehicle control; †P < 0.05 versus UA-8-treated group. (B) Histogram of the functional recovery at 40 min reperfusion expressed as percentage of baseline LVDP from C57BL/6 hearts perfused with vehicle or UA-7 (1 μM) and 14,15-EEZE (10 μM) following 20 min of ischaemia. Values represent mean ± SEM, n = 3–4 per group; *P < 0.05 versus vehicle control; †P < 0.05 versus UA-7-treated group. (C) Quantification of infarct size from the C57BL/6 hearts treated with vehicle, 11,12-EET (1 μM) and UA-8 (0.1 μM) following 20 min of ischaemia. Values represent mean ± SEM, n = 3–4 per group; *P < 0.05 versus vehicle control; †P < 0.05 versus 11,12-EET-treated group. LVDP, left ventricular developed pressure.

AKT and GSK3β, immediate downstream targets (Stephens *et al.*, 1998). Consistent with a role for PI3K, perfusion with UA-8 resulted in an increased expression of phosphorylated AKT and GSK3β in the hearts following IR compared with vehicle controls (Figure 4A,B). These data suggest the involvement of the PI3K cascade in the cardioprotective effects of UA-8. Next, we performed isolated heart experiments with the PI3K non-selective inhibitor, wortmannin and the class-I PI3K selective inhibitor, PI-103 (1 μM), to confirm the role of PI3K signalling. Perfusion with wortmannin significantly reduced the improved postischaemic functional recovery in UA-8 treated hearts. Interestingly, perfusion of PI-103 also abolished the improved postischaemic functional recovery (Figure 4C), demonstrating that class-I PI3K is involved in the UA-8-mediated cardioprotection.

Mitochondrial membrane potential ($\Delta\psi_m$)

To examine whether UA-8 affords mitochondrial protection, we conducted experiments using H9c2 cells and subjected them to 6 h anoxia followed by 16 h of reoxygenation. A marked reduction in cell death was observed following anoxia-reoxygenation in cells treated with UA-8 compared with vehicle controls (Figure 5A). Consistent with these data, cells treated with UA-8 had a lower activation of caspase-3, suggesting a reduction in anoxia-reoxygenation induced apoptosis (Figure 5B). Anoxia-reoxygenation resulted in the rapid

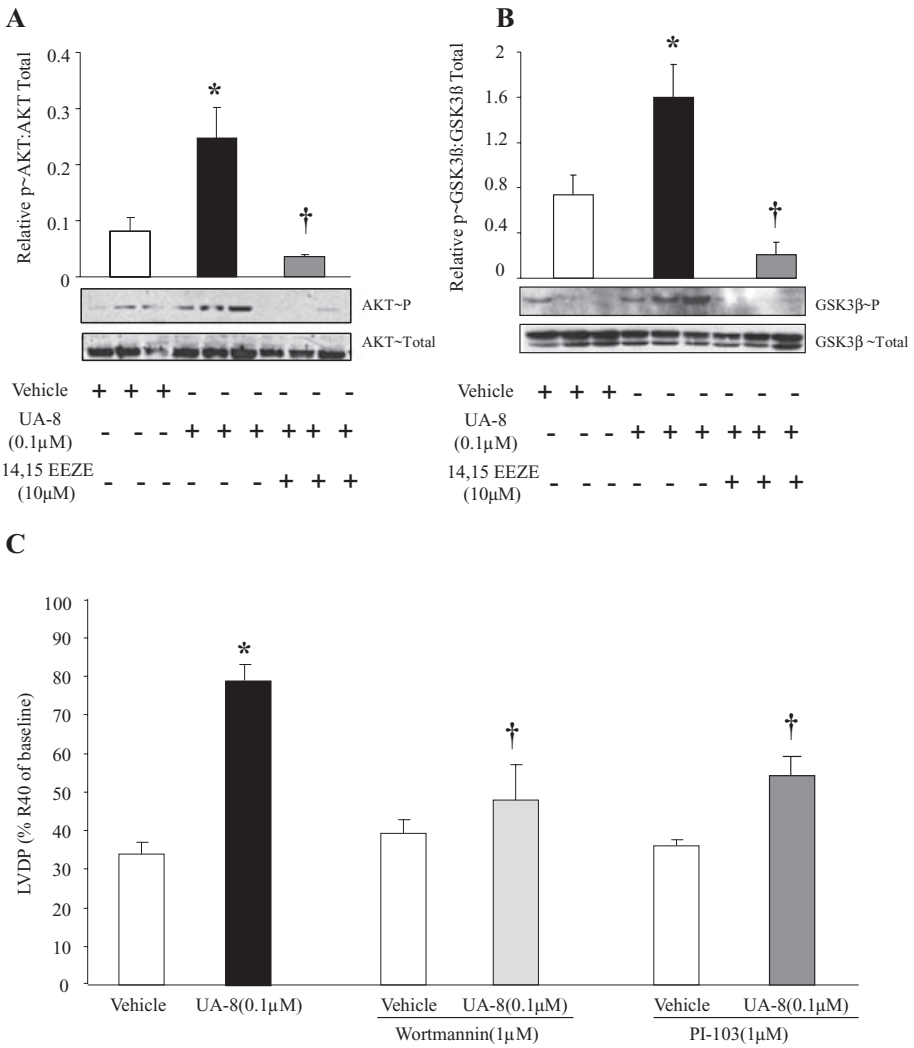

Figure 4

Role of phosphoinositide 3-kinase signalling in UA-8-mediated protection. (A) Immunoblot and densitometry showing the ratio of phospho-AKT to total-AKT expression in C57BL/6 hearts treated with vehicle, UA-8 (0.1 μM) or 14,15-EEZE (10 μM). Values represent mean ± SEM, $n = 3$ per group; *$P < 0.05$ versus vehicle control; †$P < 0.05$ versus treated group. (B) Immunoblot and densitometry showing the ratio of phospho-GSK3β to total-GSK3β expression in C57BL/6 hearts treated with vehicle, UA-8 (0.1 μM) or 14,15-EEZE (10 μM). Values represent mean ± SEM, $n = 3$ per group; *$P < 0.05$ versus vehicle control; †$P < 0.05$ versus treated group. (C) Histogram of the percentage of left ventricular developed pressure (LVDP) change at 40 min reperfusion compared with baseline from the hearts perfused with vehicle or UA-8 (0.1 μM), wortmannin (1 μM) and PI-103 (1 μM) following 20 min of ischaemia. Values represent means ± SEM, $n = 3$–6 per group; *$P < 0.05$ versus vehicle, †$P < 0.05$ versus UA-8-treated group.

dispersion of TMRE fluorescence from mitochondria (Figure 5C), indicating dissipation of $\Delta\Psi_m$ and suggesting loss of mitochondrial function. Co-treatment of cells with UA-8 significantly slowed the loss of $\Delta\Psi_{m,}$ similar to 11,12-EET-treated group. Together these data imply that UA-8 can preserve mitochondrial function caused by anoxia-reoxygenation, thereby limiting cell loss. Interestingly, when cells treated with UA-8 were co-incubated with PI-103 the protective effect was abolished (Figure 5C). These results are consistent with our functional recovery data suggesting UA-8 regulates mitochondrial function in a manner similar to EETs, and moreover, involves a class-I PI3K-mediated pathway.

Discussion

In this paper, we present data demonstrating that UA-8, a novel dual-function compound possessing both EET mimetic and sEH inhibitory properties, improves postischaemic ventricular contractile function and reduces infarct size in mouse isolated hearts. The observed cardioprotective effects were due to structural properties that mimicked EET function. Moreover, our data demonstrate that UA-8 protective mechanism(s) are mediated through a class-I PI3K-dependent pathway limiting mitochondrial damage.

EETs are chemically and metabolically labile compounds where rapid metabolism, low solubility and storage issues

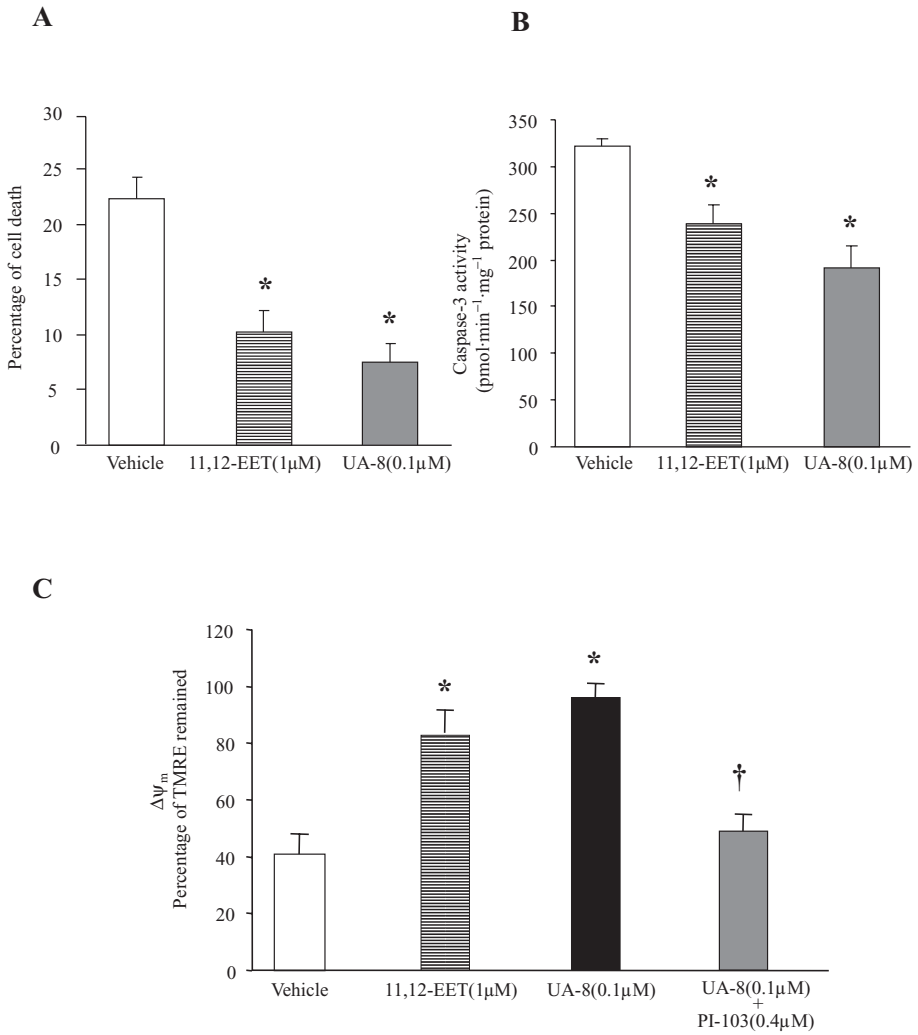

Figure 5

UA-8 limits loss of mitochondria function. (A) Cell viability: histogram representing the percentage of cells that died after being subjected to 6 h of anoxia and 16 h reoxygenation and treated with vehicle, 11,12-EET (1 μM) or UA-8 (0.1 μM). Values represent mean ± SEM; $n = 4–5$; *$P < 0.05$ versus vehicle control. (B) Caspase-3 activity in H9c2 cells using Ac-DEVD-AMC as substrate. Cells subjected to 6 h of anoxia and 16 h of reoxygenation were treated with vehicle, 11,12-EET (1 μM) or UA-8 (0.1 μM). Values represent mean ± SEM; $n = 4–5$; *$P < 0.05$ versus vehicle control. (C) Mitochondrial membrane potential ($\Delta\Psi_m$). Histograms representing the percentage of tetramethylrhodamine ethyl ester (TMRE) fluorescence lost in H9c2 cells following collapse of $\Delta\Psi_m$. Cells subjected to 6 h of anoxia and 16 h of reoxygenation were treated with vehicle, 11,12-EETs (1 μM), UA-8 (0.1 μM) or PI-103 (0.4 μM). Values indicate % change in relative fluorescence from baseline, values represent mean ± SEM; $n = 4–5$, *$P < 0.05$ versus vehicle control; †$P < 0.05$ versus treated group.

limit their use as therapeutic agents and pharmacological tools (Fitzpatrick *et al.*, 1980). Exogenously applied EETs have a rapid cellular uptake and metabolism resulting in a half-life ranging from a few seconds to minutes (Kaspera and Totah, 2009; Spector, 2009). Difficulty arises with long-term treatment using EETs as they are rapidly metabolized or incorporated into membranes (Fang *et al.*, 1997; 2004; Lee *et al.*, 1999). As such, considerable interest has arisen in developing methods to enhance the bioavailability of EETs. Decreasing the hydrolysis of EETs through inhibition of sEH using various urea-, amide- or carbamate-based pharmacological agents as an approach to elevate the EETs levels is comparable to genetic models (Jones *et al.*, 2006; Chiamvimonvat *et al.*, 2007; Motoki *et al.*, 2008; Anandan *et al.*, 2009; Xie *et al.*, 2009). Compounds such as 1,3 disubstituted urea-based old generation CDU (1-cyclohexyl-3-dodecyl-urea), AUDA (12-(3-adamantan-1-yl-ureido)dodecanoic acid), AUDA-BE (butyl ester) and new generation inhibitors like *t*AUCB with improved oral bioavailability and stability are known to be potent inhibitors of sEH (Jones *et al.*, 2006; Chiamvimonvat *et al.*, 2007; Motoki *et al.*, 2008). Recent studies have confirmed the beneficial effects of sEH inhibitors against IR injury, hypertension and stroke (Chaudhary *et al.*, 2009a; Revermann *et al.*, 2009; Qiu *et al.*, 2010). However, the drawback to these compounds as therapeutic agents includes rapid metabolism, limited oral bioavailability and low water solubility (Jones *et al.*, 2006). In addition, while these compounds succeed in inhibiting sEH and increasing EET levels,

they do not prevent EET degradation by other routes such as β-oxidation, autooxidation and esterification, and may increase EET oxidation (Falck *et al.*, 2009).

Structure activity relationship studies have demonstrated that EETs require a negatively charged group at the C-1 or need to be partially saturated with a double bond between 8, 9Δ and carbon to protect against autooxidation, as well as an epoxy group to possess EET functional effects (Falck *et al.*, 2003; 2009; Gauthier *et al.*, 2004; Yang *et al.*, 2007). Replacing the epoxy group with bioisosteres like urea, thiourea and oxiamide and partially saturating the molecule increases stability and maintains functional activity while protecting against autooxidation and sEH hydrolysis (Falck *et al.*, 2009). UA-8 is a structural analogue of EET that is partially saturated, making it less prone to oxidation, and has a 1,3-disubstituted urea replacing the epoxy group (Falck *et al.*, 2009). Recently, Falck *et al.* demonstrated that UA-8 mimics the vasodilator properties of EETs at much lower concentrations (100-fold) and exhibits resistance to oxidation in preconstricted bovine arteries (Falck *et al.*, 2009). In addition, UA-8 can inhibit sEH at nanomolar concentrations (IC_{50} 46 nM) similar to that of urea based sEH inhibitors (Falck *et al.*, 2009). While UA-8 can potentially undergo β-oxidation, an increased resistance to autooxidation, esterification and degradation provides a significant advantage compared with other sEH inhibitors leading to a longer duration of its protective action. In the present study, we demonstrated that hearts perfused with UA-8 at concentrations as low as 100 nm show significant improvement in LVDP preventing the loss in cardiac contractile function, similar to the cardioprotection elicited by EETs (1 μM) or sEH null mice. Moreover, UA-8 significantly reduced the amount of irreversible injury, or infarction, observed in control and EET-treated hearts. Thus demonstrating UA-8 has better potency compared with EETs alone, most likely reflecting increased stability. Improved recovery following perfusion with UA-7, which has a sEH IC_{50} of 58 712 nM, suggested that the protective effect of UA-8 was attributed to EET mimetic properties as opposed to sEH inhibition (Falck *et al.*, 2009). This finding was further supported when UA-8 and UA-7 improved postischaemic recovery was attenuated by the EET-antagonist, 14,15-EEZE and the lack of effect of MS-PPOH. Thus these data suggest that EET functional properties are important for limiting ischaemia-reperfusion injury.

Previous studies have shown EET-mediated cardioprotection involves activation of a PI3K-mediated pathway phosphorylating downstream targets like AKT and GSK3β (Seubert *et al.*, 2006; Dhanasekaran *et al.*, 2008; Chaudhary *et al.*, 2009b). PI3Ks are members of a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphoinositides. PI3K phosphorylates phosphoinositol-3,4-bisphosphate (PIP_2) producing phosphoinositol-3,4,5-triphosphate (PIP_3), considered important lipid messenger molecules that regulate the localization and function of multiple proteins and ion channels (Suh and Hille, 2008). Enhanced activation of PI3Ks and signalling pathways involving downstream kinases in IR is known to reduce cell death and infarct size (Murphy *et al.*, 2003). Consistent with reports of EET-mediated cardioprotection, hearts perfused with UA-8 had increased expression of activated AKT and GSK3β (Seubert *et al.*, 2006; Dhanasekaran *et al.*, 2008; Chaudhary *et al.*, 2009b). Thus, the current results suggest that UA-8 reproduces the EETs protective mechanism and support the involvement of PI3K survival signalling. PI3Ks are classified into various classes (class-I, -II and -III) based on mode of activation and structure. PI3Ks from all classes produce the same secondary messenger but are known to have a different mechanism of action (Oudit and Penninger, 2009). PI3Ks studies often use non-specific inhibitors, like wortmannin, making it difficult to differentiate which isoform is responsible for the protective effect. Our data demonstrate that the class-I specific PI3K inhibitor, PI-103, abolished the UA-8 protective action, suggesting a role for these kinases in the protective response.

Mitochondria are strategic regulators of life and death, playing a central role in energy production, calcium homeostasis and programmed cell death. Ischaemic injury causes distinct morphological changes to mitochondria impacting dynamics and function (Chan, 2006). Activation of PI3K initiates survival pathways that can prevent the collapse of mitochondrial function following injury (Tong *et al.*, 2002; Juhaszova *et al.*, 2004). The $\Delta\Psi_m$, a marker of mitochondrial activity, can provide information about cellular function following significant cell stress conditions and loss of $\Delta\Psi_m$ ultimately contributes to contractile dysfunction and cell death (Juhaszova *et al.*, 2004). In the present study, incubation of H9c2 cells with UA-8 slowed the loss of $\Delta\Psi_m$ following anoxia-rexoygenation injury. Importantly, the effects of UA-8 were blocked by co-administration of PI-103, suggesting a role for class-I PI3K isozymes in maintaining mitochondrial function following cellular injury. In addition, UA-8 treatment reduced activation of caspase-3 activity limiting the apoptotic cell death attributed to an IR injury.

In summary, we report improved postischaemic contractile function and reduced infarct size with the dual-action compound, UA-8, that possesses EET mimetic and sEH inhibitory properties. Moreover, our data suggest that the EET mimetic properties are important for the improved recovery and class-I PI3K are involved in the protective mechanism. The increased potency and stability of UA-8 may serve as a potential therapeutic agent in limiting mitochondrial damage and myocardial injury attributed to ischaemia/reperfusion events.

Acknowledgements

The authors would like to thank Glenis Wiebe for reviewing the manuscript. J. M. S. is the recipient of a New Investigator Award from the Heart and Stroke Foundation of Canada and a Health Scholar Award from the Alberta Heritage Foundation for Medical Research for all the valuable assistance. This work was supported by Canadian Institutes of Health Research Grant (J. M. S. MOP79465). The work was supported in part by the United States Public Health Service National Institutes of Health (J. R. F. GM31278) and the Robert A. Welch Foundation (J. R. F. GL625910).

Conflict of interest

None declared.

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