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Original article

Soluble epoxide hydrolase activation by *S*-nitrosation contributes to cardiac ischemia–reperfusion injury

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ABSTRACT

Cardiac ischemia–reperfusion (I/R) injury always accompanies recanalization treatment for myocardial infarction. Here we found soluble epoxide hydrolase (sEH), which metabolizes cardioprotective epoxyeicosatrienoic acids into less effective diols, was rapidly activated during myocardial reperfusion in both mouse and rat models in expression-independent manner. Similar activation was mimicked by nitric oxide (NO) donor dose-dependently *in vitro*, along with an obvious induction of sEH *S*-nitrosation, a short-term post-translational modification, which diminished in sEH Cys-141-Ala mutant. *In vivo*, I/R induced sEH *S*-nitrosation could be reversed by NO synthase inhibitor L-NAME, with protective effect on cardiac dysfunction, which however vanished in sEH$^{-/-}$ mice. Further, a protective effect against I/R injury in the initial phase of reperfusion was observed in eNOS$^{-/-}$ mice, indicating inhibition of NO as a sEH-based cardioprotective in early time of I/R injury. Besides, sEH inhibitor directly targeting on activated sEH during cardiac reperfusion significant reduced infarct size after I/R *in vivo*. In summary, our findings show the critical role of sEH *S*-nitrosation in cardiac I/R injury and inhibiting sEH *S*-nitrosation may be a new therapeutic strategy clinically.

1. Introduction

Acute myocardial infarction (AMI) is a major and common fatal ischemic cardiovascular disease that often results from coronary artery stenosis and leads to regional or global cardiomyocyte death. Ischemia–reperfusion (I/R) injury may occur as a side effect of percutaneous coronary intervention (PCI) or thrombolysis therapy for AMI. A fundamental mechanism of I/R is the disruption of redox balance, whereby reactive nitrogen species – and reactive oxygen species (ROS) – induced post-translational modification (PTM), such as tyrosine nitration [1], cysteine *S*-nitrosation [2] and lipid peroxidation [3], can trigger pro- or anti-apoptosis signaling pathways [4,5]. *S*-Nitrosation is a reversible redox-dependent coupling of an NO moiety to a reactive cysteine thiol to form an *S*-nitrosothiol (SNO). In general, *S*-nitrosation can rapidly regulate protein activity or subcellular localization [6], interaction with other proteins, which is an important mechanism of NO-mediated signal transduction.

Soluble epoxide hydrolase (sEH) catalyzes the conversion of epoxyeicosatrienoic acids (EETs), cardioprotective arachidonic acid metabolites, to their corresponding dihydroxyeicosatrienoic acids (DHETs) and reduces their bioactivity [7]. Previous studies demonstrated that increased sEH activity is related to several systemic diseases such as cardiac hypertrophy, hypertension and obesity [8,9]. Biochemical inhibition or gene deletion of sEH could diminish the size of MI [10]. A major protective mechanism is that sEH inhibition increases EETs, the well-known vasodilators, therefore activates cellular protective pathways

Abbreviations: A/R, Anoxia–reoxygenation; A3R3, 3 h of anoxia followed by 3 h of reoxygenation; AAR, Area at risk; AMI, Acute myocardial infarction; C-to-A, Cysteine-to-alanine; CysNO, *S*-nitrosocysteine; DHETs, Dihydroxyeicosatrienoic acids; EETs, Epoxyeicosatrienoic acids; EF, Ejection fraction; FS, Fractional shortening; I/R, Ischemia–reperfusion; IBP, Irreversible Biotin-switch Procedure; LCA, Left coronary artery; LV, Left ventricular; PCI, Percutaneous coronary intervention; PTM, Post-translational modification; RNCMs, Rat neonatal cardiac myocytes; ROS, Reactive oxygen species; SD, Sprague–Dawley; sEH, Soluble epoxide hydrolase; SNO, *S*-nitrosothiol; TUPS, 1-[1-methanesulfonyl-piperidin-4-yl]-3-[4-trifluoro-methoxy-phenyl]-urea; UPLC-MS/MS, ultra-performance liquid chromatography-mass spectrum/mass spectrum.

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[11]. EETs concentration was reported to be decreased in an I/R dog model, accompanied by increased DHETs [12]. These data indicate the involvement of spontaneous upregulation of sEH activity in the reperfused heart. Several previous reports suggest that sEH inhibitors prophylactically protect the heart against I/R injury [13,14], few studies have examined the therapeutic effect on the mechanism behind increased sEH activity following I/R injury to the heart, while this study goes into greater detail.

sEH is a homodimeric domain-swapped architecture protein containing 2 function domains: a hydrolase domain in the C-terminus and a phosphatase domain in the N-terminus [15]. Catalysis central amino acids, including Y383, Y466 and C523 in human sEH, are involved in ischemia-induced sEH PTM and lead to hydrolase inhibition [1,3,16]. A number of single nucleotide polymorphisms in human sEH, such as R103C, R287Q, K55R and C154Y, can alter its activity [17]; some have been linked to coronary heart disease [18]. Thus, conservation of certain amino acids may be necessary for sEH activity, which implies PTM-induced sEH activation in I/R injury.

After the stable isotope labeling by amino acids in cell culture proteomic study indicated sEH as a potential endogenous protein target of *S*-nitrosation [19], we hypothesized that sEH activity might be regulated in the I/R heart by *S*-nitrosation. We found a relationship between sEH activity and sEH-SNO protein level, which is correlated with myocardial injury. Moreover, the sEH inhibitor 1-[1-methanesulfonyl-piperidin-4-yl]-3-[4-trifluoro-methoxy-phenyl]-urea (TUPS) could therapeutically attenuate cardiac I/R injury by inhibiting sEH activity.

2. Materials and methods

Detailed information has been provided in an online supplementary material.

2.1. Rat and mouse models of myocardial I/R

Experimental procedures were approved by the Peking University Institutional Animal Care and Use Committee (LA2011–003). All animal experiments were carried out according to the *Guide for the Care and Use of Laboratory Animals:* Eighth Edition (US National Research Council). Male Sprague–Dawley (SD) rats weighing 250–300 g (8 weeks old) and male 7- to 9-week-old C57BL/6J, eNOS$^{-/-}$ and sEH$^{-/-}$ mice and their littermate wild-type controls were used for left coronary artery (LCA) ligation surgery. To determine the amount of rat myocardial infarction and area at risk size, after 2-h reperfusion, the LCA was re-occluded, the infarct size was quantified as described in Supplementary data.

2.2. Anoxia–reoxygenation (A/R) model of rat neonatal cardiac myocytes (RNCMs)

Primary cultures of RNCMs were generated and cultured as described [21]. Anoxia of cells was performed in an air tight jar (Thermo Fisher, USA) by flushing out the standard gas mixture containing 5% CO_2 and 95% N_2 for 1 or 3 h. The culture medium was changed to serum-free, low-glucose DMEM before A/R. The reoxygenation involved exchanging the culture medium with fresh high-glucose DMEM with 10% FBS and culturing in normoxic air (5% CO_2, 20% O_2 and 75% N_2) for another 3 or 6 h to recover both the supply of oxygen and nutrition. Human sEH adenovirus (Ad-sEH) or control adenovirus (green fluorescence protein, Ad-GFP) was amplified, and cells were transfected 1 day before A/R as described [44].

2.3. Assays of sEH activity

For UPLC-MS/MS measurement of sEH activity, EET/DHET were measured as previously reported [45]. Plasma and cell culture medium samples were spiked with 1 μl internal standard mixture (5 ng for each internal standard). Heart tissue was extracted by methanol and ethyl acetate successively. The Waters Oasis-HLB cartridges (1 ml volume) were used for solid-phase extraction of plasma or medium. Heart tissue extraction solution and solid-phase extraction elution were further dried and re-dissolved in 30% acetonitrile and filtered. Ultra-high-performance liquid chromatography (UPLC, Waters, Milford, MA) involved use of a 5500 QTRAP hybrid triple-quadruple linear ion trap mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo Ion Spray electrospray ionization source (UPLC-MS/MS) as described [44].

2.4. Protein S-nitrosation detection with Irreversible Biotin-switch Procedure (IBP)

Analysis of sEH-SNO with the IBP in H9c2 and HEK293 cells and heart tissues was performed as described [46]. The largest part of the IBP procedure is under indirect light. This method was improved from the original biotin-switch assay [47] and described in Supplementary data.

2.5. Determination of NO generation in rat cardiac tissues

40 mg rat cardiac tissues were homogenized in 1 ml lysis buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 1% NP40 with complete protease inhibitor cocktail). After centrifugation, 50 μl lysates were incubated with 100 μl Griess reagent (equal volume of 1% sulfanilamide in 0.1 M HCl and 0.1% N-[-1-naphthyl-ethylenediamine dihydrochloride]) (Beyotime Biotechnology). Nitrite concentrations were determined from a standard curve (0–20 μM) by spectrophotometry at absorbance wavelengths of 560 nm. Data were expressed as mean ± SEM.

2.6. Statistical analysis

In all groups, data are expressed as mean ± SEM from at least 3 independent experiments. Unpaired two-tailed Student's *t*-test and one or two-way ANOVA were performed to compare different groups. Each experiment included duplicated measurements for each condition tested, unless indicated otherwise. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cardiac I/R induced sEH activity both in vivo and in vitro

To investigate the regulation of sEH activity during reperfusion period in cardiac tissue, we established the *in vivo* I/R model by surgery in Sprague-Dawley rats. The left ventricle was made locally ischemic for 30 min followed by 2-h reperfusion. S-T segment elevation in ECG indicated successful cardiac ischemia or infarct (Supplementary Fig. 1a); arrhythmia, such as ventricular tachycardia (Supplementary Fig. 1a,b), indicated coronary artery reperfusion. sEH protein expression in the area at risk (AAR) of the heart remained unchanged after 10, 60 or 120 min of reperfusion (Fig. 1a), but sEH activity in the AAR was significantly increased by ~2-fold using the catalytic fluorescence assay after 2 h of reperfusion (Fig. 1b), and hydrolysis of 5,6-, 11,12- and 14,15-EET regioisomers was decreased by ~50% detected with ultra-performance liquid chromatography-mass spectrum/mass spectrum (UPLC-MS/MS); 8,9-EET showed similar trend without significance (Fig. 1c). To further support the alteration in sEH activity, DHET/EET ratios in both the AAR and plasma of rats with reperfusion were also increased, especially the 14,15-DHET/EET ratio, which indicates enhanced EET degradation (Fig. 1d,e).

Next, we isolated rat neonatal cardiomyocytes (RNCMs) to establish an *in vitro* reoxygenation injury model. Anoxia for 3 to 6 h followed by 1 or 3 h of reoxygenation successfully induced RNCM cell death (Supplementary Fig. 1b) and significantly upregulated the protein expression of