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Soluble epoxide hydrolase inhibition exerts beneficial anti-remodeling actions post-myocardial infarction

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ABSTRACT

Background: A contributory role for soluble epoxide hydrolase (sEH) in cardiac remodeling post-myocardial infarction (MI) has been suggested; however effects of sEH inhibition following MI have not been evaluated. In this study, we examined in vivo post-MI anti-remodeling effects of a novel sEH inhibitor (GSK2188931B) in the rat, and evaluated its direct in vitro effects on hypertrophy, fibrosis and inflammation.

Methods and results: Post-MI administered GSK2188931B (80 mg/kg/d in chow) for 5 weeks improved left ventricular (LV) ejection fraction compared to vehicle-treated (Veh) rats ($P<0.01$; Sham 65 ± 2%, MI + Veh 30 ± 2%, MI + GSK 43 ± 2%) without affecting systolic blood pressure. Percentage area of LV tissue sections stained positive for picrosirius red (PS) and collagen I (CI) were elevated in LV non-infarct zone ($P<0.05$; NIZ; PS: Sham 1.46 ± 0.13%, MI + Veh 2.14 ± 0.22%, MI + GSK 1.28 ± 0.14%; CI: Sham 2.57 ± 0.17%, MI + Veh 5.06 ± 0.58%, MI + GSK 2.97 ± 0.34%) and peri-infarct zone ($P<0.001$; PIZ; PS: Sham 1.46 ± 0.13%, MI + Veh 9.06 ± 0.48%, MI + GSK 6.31 ± 0.63%; CI: Sham 2.57 ± 0.17%, MI + Veh 10.51 ± 0.64%, MI + GSK 7.77 ± 0.57%); GSK2188931B attenuated this increase ($P<0.05$). GSK2188931B reduced macrophage infiltration into the PIZ ($P<0.05$). GSK2188931B reduced AngII- and TNFα-stimulated myocyte hypertrophy, AngII- and TGFβ-stimulated cardiac fibroblast collagen synthesis, including markers of gene expression ANP, β-MHC, CTGF and CI ($P<0.05$). GSK2188931B reduced TNFα gene expression in lipopolysaccharide (LPS)-stimulated monocytes ($P<0.05$).

Conclusion: sEH inhibition exerts beneficial effects on cardiac function and ventricular remodeling post-MI, and direct effects on fibrosis and hypertrophy in cardiac cells. These findings suggest that sEH is an important contributor to the pathological remodeling following MI, and may be a useful target for therapeutic blockade in this setting.

1. Introduction

Left ventricular systolic dysfunction following acute myocardial infarction (MI) is associated with high short-term morbidity and mortality as well as with ongoing pathological remodeling and ultimately the clinical syndrome of heart failure. In this regard, strategies to ameliorate this remodeling process are of major clinical interest and importance. However, despite current therapies, morbidity and mortality remain unacceptable and new potential targets are urgently required to reduce the short, medium and long-term effects of the left ventricular systolic dysfunction that follows MI.

Epoxyeicosatrienoic acids (EETs) are arachidonic acid metabolites of the CYP450 pathway that possess vasodilatory, anti-inflammatory and anti-hypertrophic properties [1,2]. Beneficial cardiovascular properties of EETs have been demonstrated in experimental models of disease. EET levels are limited by soluble epoxide hydrolase (sEH) mediated catalysis to their corresponding dihydroxyeicosatrienoic acids (DHETs). An effector enzyme, sEH was originally described in angiotensin-II mediated vasoconstriction and hypertension, and the use of sEH inhibitors demonstrated their ability to reduce blood pressure in spontaneously hypertensive rats and in rats infused with angiotensin II [3,4]. Furthermore, administration of exogenous EETs has been shown to produce a marked reduction in infarct size in canines [5]. Ex-vivo studies in mice have demonstrated significant cardioprotective effects of EETs against ischemia-reperfusion injury involving the PI3K pathway [6,7].

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More recently, the use of sEH knockout mice and selective sEH inhibitors has demonstrated a potential hypertrophic role for sEH in the myocardium [8–11]. It is still unclear, however, how much of the anti-hypertrophic effects of sEH inhibitors are mediated via reductions in after-load on the heart, or are a direct result of inhibition of myocyte hypertrophy. In support of the latter proposition, a significant reduction in cell size in cardiac myocytes treated with sEH inhibitors has been observed in response to in vitro angiotensin-II stimulation [8,11]. In rodent cardiac myocytes over-expressing sEH, elevated enzyme activity and increased hypertrophy were accompanied by elevated gene expression of hypertrophic markers ANF and βMHC, whereas these effects were abolished in sEH knockout mice [8].

Soluble EH inhibitors have been examined over the past decade for their therapeutic applications in cardiovascular disease. The effect of sEH inhibition on MI-induced cardiac remodeling, administered following permanent coronary artery occlusion has not previously been examined. In this study the sEH inhibitor GSK2188931B (N-({4-bromo-2-[(trifluoromethyl)oxy]phenyl}methyl)-1-[4-methyl-6-(methylamino)-1,3,5-triazin-2-yl]-4-piperidinecarboxamide) was administered post-MI, and the effects on cardiac function and structure as well as gene expression were examined. In addition, we evaluated the direct effects of sEH inhibition on cardiac cells, specifically myocytes and fibroblasts, to determine effects on cardiac hypertrophy and collagen synthesis, respectively. Inflammatory cells post-MI have been found to be critical to remodeling processes [12,13]; hence we also examined the effect of sEH inhibition on cytokine gene expression using a monocytic cell line.

2. Methods

2.1. Rat model of myocardial infarction and study design

Male Sprague–Dawley rats (approximately 250 g) were anesthetized with Alfaxan (15 mg/kg, iv), intubated and maintained on 1% isoflurane (in oxygen, ih). Permanent ligation of the left descending coronary artery was performed as previously published [14]. Depth of anesthesia was monitored by reflex response to toe-pinch. Following surgery buprenorphine (Temgesic 0.01 mg/kg, sc) was administered for analgesia. Sham animals underwent the same procedure except the coronary artery was not tied.

On waking, MI animals were randomized to receive vehicle (standard rat chow) or GSK2188931B (1000 ppm in chow; approximately 80 mg/kg/d) for 5 weeks. Sham animals received standard rat chow for 5 weeks. At the conclusion of the study, animals underwent assessment of cardiac function by echocardiography and cardiac catheterization for pressure–volume loop analysis, and cardiac tissue was harvested at sacrifice for histological and molecular investigations. Plasma was obtained at 5 weeks for determining levels of GSK2188931B and its bioactivity, in particular the endogenous sEH substrate to product ratio, leukotoxin/leukotoxin diol.

These studies conform to the Australian code of practice for the care and use of animals for scientific purposes 7th edition 2004, and were approved by the Animal Ethics Committees of Monash University and St Vincent's Hospital.

2.2. Echocardiography

Transthoracic echocardiography was performed as previously described [15] after 5 week treatment using a Vivid 7 (GE Vingmed, Horten, Norway) echocardiography machine with a 10 MHz phased array probe. Parasternal short-axis views of the heart were used to obtain measures of LV internal dimension in diastole (LVIDd) and systole (LVIDs), and LV posterior (LVPWd) and anterior (LVPWd) wall thicknesses in diastole. Parasternal long-axis views were used to obtain LV end diastolic (LVEDV) and end systolic (LVESV) volumes. Ejection fraction (EF) and percent fractional shortening (%FS) were calculated according to standard formulae [16]. All parameters were assessed using an average of three beats and calculations were made in accordance with the American Society of Echocardiography guidelines [16]. All data were acquired and analyzed by a single blinded observer using Echo PAC (GE Vingmed) off-line processing.

2.3. Cardiac catheterization

Hemodynamics were performed as previously described [15], briefly, a 2F miniaturized combined catheter/micromanometer (Model SPR838 Millar instruments, Houston, TX) was inserted into the right common carotid artery to obtain aortic blood pressures, and then advanced into the left ventricle to obtain left ventricular pressure–volume (PV) loops. PV loops were recorded at steady state and during transient preload reduction, achieved by occlusion of the inferior vena cava and portal vein with the ventilator turned off and animal apnoeic. The following validated parameters were assessed using Millar conductance data acquisition and analysis software PVAN 3.2: left ventricular end diastolic pressure (LVEDP), the maximal rate of pressure rise (dP/dt_{max}) and fall (dP/dt_{min}), the slope of the end systolic pressure–volume relationship (ESPVR), the slope of the end diastolic pressure–volume relationship (EDPVR), Tau (t Logistic), and the slope of the preload recruitable stroke work (PRSW) relationship.

2.4. Quantification of plasma GSK2188931B, leukotoxin and leukotoxin diol levels

Analysis of rat plasma samples for GSK2188931B and the sEH mechanistic biomarkers (substrate/product) leukotoxin/leukotoxin diol and 14,15-EET/DHET was performed using liquid chromatography/tandem mass spectrometric (LC–MS/MS) detection. The samples were thawed, plasma proteins precipitated with an appropriate volume of 95/5 acetonitrile/0.1% aqueous formic acid containing two mass spectral internal standards, and the resulting mixture vortex-mixed followed by centrifugation. A matrix matched standard curve was prepared for GSK2188931B and the biomarkers to confirm sensitivity and linearity of the instrument response. The resulting supernatant was injected onto the LC–MS/MS system and eluted via a gradient profile. A positive-ion ESI (electron-spray ionization) multiple reaction monitoring method for GSK2188931B was characterized by the transition of the m/z 503.3/206.1. Similarly, the biomarkers were analyzed using a negative-ion ESI multiple reaction monitoring method characterized by the following transitions: leukotoxin (m/z 295.2/277.4), leukotoxin diol (m/z 313.5/201.2), 14,15-EET (m/z 319.1/219.0), and 14,15-DHET (m/z 337.3/206.9). Data for the analytes (GSK2188931B and the biomarkers) were reported as quantitative drug concentrations as determined by plotting the analyte/internal standard peak area ratios versus analyte concentration. Since the biomarkers were endogenous and at quantifiable levels in the matrix used in the standard curve, the method of standard additions was used to compensate for their background concentrations.

2.5. Histology and immunohistochemistry

Sections (4 μm) were stained with hematoxylin and eosin to determine infarct size and myocyte cross sectional area. Infarct size was calculated from images obtained under a low magnification microscope and infarct size expressed as an averaged percentage of the endocardial and epicardial scarred circumferences of the LV using image analysis software Analytical Imaging Station (AIS, Version 6.0, Ontario, Canada). Animals with small infarcts were omitted from the analysis; small infarcts were classified as being <25% of the left ventricular circumference determined from hematoxylin and eosin stained sections. For myocyte cross-sectional area, images were digitally captured using an AxioImager.A1 microscope (Carl Zeiss AxioVision) attached to an AxioCam MRc5 digital camera (Carl Zeiss AxioVision) at 400× magnification. Myocytes in the same plane, as assessed by similar sized nuclei, were outlined and the area measured from 50 myocytes with intact cellular membranes per LV. The circumferences were traced to calculate mean cross-sectional area using image analysis software (AIS).

Sections were stained with picrosirius red to compare regional fibrosis within the myocardium, in particular the non-infarct zone (NIZ; sub-endocardial region remote to the infarct), peri-infarct zone (PIZ; region bordering the infarct and non-infarct zones) and the infarct zone (IZ; within the scarred area). Antibody staining of LV tissue sections for collagen I (Southern Biotech, Birmingham, AL, USA), collagen III (BioGenex, San Ramon, CA, USA) and the macrophage marker CD68 (Serotec, Raleigh, NC, USA) was assessed by positive diaminobenzidene staining as previously described [17]. Positive staining was quantified by a single blinded researcher using AIS software (Version 6, Imaging Research Inc). Results were expressed as average percentage area of 10 random fields at 100× magnification for each zone (NIZ, PIZ, IZ) in each section obtained in the subendocardial region of the LV. In sham animals there was no PIZ or IZ, hence only NIZ was measured. For quantitation of macrophage infiltration, interstitial macrophages were identified and individually counted under the microscope at 400× magnification and expressed as number/mm^2 [18].

2.6. Quantitation of left ventricular mRNA expression

Total RNA was extracted from frozen NIZ and PIZ tissue using Ambion mini-kits (Ambion, Austin, TX, USA). Total RNA was reverse transcribed and real time PCR performed in triplicate using sequence-specific primers (Geneworks, Adelaide, SA, Australia) and a TaqMan fluorogenic probe (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed and quantified for mRNA expression of β-MHC, ANP, $TGF\beta_1$, CTGF, procollagen-α_1(I), and GAPDH using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The primer pairs and probes were designed using Primer Express 2.0 software (Applied Biosystems) based on published sequences (http://www.ncbi.nlm.nih.gov) as previously described (Table 1) [19]. β-MHC, ANP and GAPDH primer and probe combinations were purchased from Applied Biosystems Assays-on-Demand.