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Epoxyeicosatrienoic acids and cardioprotection: the road to translation

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1. Introduction

Despite major advances in evidence-based medical therapies, cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide. In the western world, CVD has been the leading cause of death for almost a century and its prevalence is expected to continue to rise tremendously [1, 2]. Most notably, acute myocardial infarction (AMI) events, complications of CVD, are a primary source of the public health burden associated with this illness [1, 2]. AMI is typically characterized by rupture of an atheromatous plaque resulting in an intracoronary thrombus and myocardial ischemia [3]. The restoration of blood flow, termed ischemia-reperfusion (IR), is imperative to prevent further myocardial cell necrosis. Paradoxically, however, IR also triggers injury to the myocardium [4]. Consequently, identification and characterization of the key pathways that regulate IR injury will facilitate the development of novel therapeutic strategies that mitigate IR injury and its pathological consequences, thereby reducing the risk of adverse outcomes following AMI.

It is now well-established that cytochrome P450 (CYP)-derived epoxyeicosatrienoic acids (EETs), endogenous lipid metabolites of arachidonic acid, elicit potent anti-inflammatory, vasodilatory, fibrinolytic, anti-apoptotic, pro-angiogenic, and smooth muscle cell anti-

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migratory effects in the cardiovascular system [5, 6]. Furthermore, accumulating preclinical evidence from *in vitro*, *ex vivo*, and *in vivo* models of AMI demonstrate that EETs directly protect the myocardium following ischemia via a variety of mechanisms [7–9]. Additionally, associations between genetic polymorphisms in the CYP epoxygenase pathway and the risk of developing CVD have been reported in humans [10]. Therefore, therapeutic interventions that promote the cardioprotective effects of EETs offer considerable promise as a novel therapeutic strategy to reduce sequelae following AMI; however, key questions remain to be addressed prior to translation of EET-promoting strategies into successful proof-of-concept phase I and II clinical trials. The acute and chronic cardioprotective effects of EETs and underlying mechanisms have not been fully characterized. Furthermore, the association between genetic polymorphisms in the CYP epoxygenase-EET pathway and poor prognosis has not been studied in patients suffering from an AMI. These are currently active areas on investigation.

This review aims to 1) outline the known cardioprotective effects of EETs and underlying mechanisms with a particular focus on myocardial IR injury, 2) describe studies in human cohorts that demonstrate a relationship between EETs and associated pathways with the risk of coronary artery disease (CAD), and 3) discuss preclinical and clinical areas that require further investigation in order to increase the probability of successfully translating this rapidly emerging body of evidence into a clinically applicable therapeutic strategy for AMI.

2. The CYP epoxygenase pathway

Arachidonic acid is metabolized by CYP epoxygenase enzymes to form bioactive EETs (Fig. 1) [11]. CYP2J and CYP2C epoxygenases are the primary source of all four EET regioisomers (5,6-, 8,9-, 11,12-, and 14,15- EETs) [12]. Each regioisomer is composed of 2 different stereoisomers (R,S or S,R configuration) [12]. CYP2J2, CYP2C8 and CYP2C9 are extensively and constitutively expressed in human heart tissue [13, 14]. The predominant fate of EETs is through rapid metabolism by soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acids (DHETs), which generally have less biological activity [6, 7]. *EPHX2* codes for human sEH [15] and is expressed in a multitude of cell types [16]. Importantly, sEH is highly expressed in the myocardium [16].

In parallel, arachidonic acid is also metabolized by cyclooxygenase, lipoxygenase and CYP hydroxylase enzymes to produce biologically active metabolites that play a functional role in myocardial IR injury [17–19]. In addition to arachidonic acid-derived products, other members of the n-6 polyunsaturated fatty acid (PUFA) family (most notably linoleic acid) and of the n-3 PUFA family such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) play a role in cardiovascular disease [20]. CYP-dependent epoxy-derivatives of these PUFAs are also potent biological mediators in the cardiovascular system and may be subsequently metabolized into vicinal diols by epoxide hydrolases [12, 21, 22]. Although these emerging data are beyond the scope of this review, we summarize select examples from the literature throughout the review that will stimulate future research in this area.

A variety of pharmacologic and genetic strategies have been utilized to characterize the functional role of EETs in preclinical studies. Administration of exogenous EETs and

synthetic EET analogs has been utilized as agonists to characterize the direct cardioprotective effects of EETs [23]. The synthetic analog 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) has the unique property of exhibiting putative EET receptor antagonist-like activity [24], and consequently has also been very useful to study EET action. An alternative approach is to target enzymes involved in the biosynthesis (CYP epoxygenases) and metabolism (sEH) of endogenous EETs. Notably, cardiomyocyte-specific CYP2J2 overexpression (αMHC-CYP2J2-transgenic [Tr] mice), global disruption of *Ephx2* (*Ephx2*$^{-/-}$ mice), and pharmacologic inhibition of sEH have each been utilized to increase EETs *in vivo* and study the contribution of the CYP epoxygenase-EET pathway to cardioprotection [25–27].

3. Acute EET effects following IR

Accumulating evidence, across multiple laboratories and species, has demonstrated that EETs abrogate a variety of acute pathophysiological responses following myocardial IR, including the reduction of left ventricular infarct size and improved recovery of left ventricular function (Table 1) [8]. Furthermore, as outlined in more detail below, EETs elicit these cardioprotective effects through multiple mechanisms, namely through activation of prosurvival signaling, attenuation of apoptosis, and promotion of mitochondrial protection acutely following IR (Table 1, Fig. 2B).

3.1 Promotion of prosurvival signaling

Numerous signal-transduction pathways are activated following IR, dictate the extent of cell survival following myocardial injury, and thus are pro-survival therapeutic targets for cardioprotection [28, 29]. Our group has shown that αMHC-CYP2J2-Tr mice exhibit increased myocardial DHET biosynthesis (the stable metabolite of EETs) and improved left ventricular developed pressure (LVDP) following 20 minutes of ischemia and 40 minutes of reperfusion compared to wild-type littermate controls [30]. This cardioprotection was thought to be mediated by putative mitochondrial K_{ATP} (mitoK_{ATP}) channel-derived and p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) prosurvival signaling [30]. The exogenous administration of 11,12-EET produced a similar recovery of ventricular function [30]. Further evidence of improved recovery of LVDP following IR was observed in *Ephx2*$^{-/-}$ mice, which was reversed by 14,15-EEZE implicating that the effect was promulgated directly by EETs. Infarct size was also reduced in these mice [31]. These actions were mediated via activation of sarcolemma K_{ATP} (sarcK_{ATP}) and mitoK_{ATP} channels as well as phosphatidylinositol-3 kinase (PI3K) signaling [31]. Results in canine and rat models of AMI have confirmed the role of K_{ATP} channels in EET-mediated cardioprotection [32–34].

Further research has brought the role of prosurvival signaling in EET-mediated cardioprotection to a more detailed level of understanding. Evidence suggests the timing of EET administration relative to IR injury is important and that EET-mediated cardioprotection is regulated by the activation of sarcK_{ATP} channels only if EETs are administered during the ischemic period [35]. Selective inhibition of PI3K was found to attenuate improved LVDP and infarct size from an agent that possesses both EET-mimetic and sEH inhibitory properties, implicating class-I isozymes of PI3K in EET-mediated

cardioprotection [36]. Moreover, phosphatidylinositol-3 kinase-alpha (PI3Kα) was reported to be the specific isoform within the class-I PI3K family that was implicated in EET-mediated protection [37]. Interestingly, these effects occurred through a PI3Kα-dependent activation of sarcK_{ATP} channels [37]. Results from another study conflict with this data by showing that EETs activate sarcK_{ATP} channels independent of PI3K-mediated pathways [38]. Experimental factors such as species (rats versus mice), EET regioisomer (14,15-EETs versus 11,12 EETs), and inhibitor (wortmannin versus PI-103) may explain these discordant findings. Indeed, evidence from a study that improved LVDP following IR injury with 11,12-EET that was absent in 14,15-EET at the same dose suggests that the cardioprotective potency of EETs may vary by regioisomer [39]. Studies have also implicated endothelial NO synthase (eNOS) [35], signal transducer and activator of transcription 3 (STAT3) [40], brain natriuretic peptide (BNP) [41], and opioid receptor [42] signaling in EET-mediated cardioprotection. Overall, work remains necessary to further delineate the relative contribution of specific signaling pathways and regioisomers to the cardioprotective effects of EETs.

3.2 Attenuation of apoptosis

It is well-established that activation of apoptosis promotes infarct size and worsens recovery of cardiac function following IR injury [43]. Importantly, EETs possess potent anti-apoptotic properties in cardiomyocytes. Cultured cells from neonatal rat hearts and a mouse atrial lineage (HL-1) that were pretreated with EETs had reduced expression of multiple markers of apoptosis and maintained rhythmic myocyte beating after 8 hours of hypoxia and 16 hours of reoxygenation [44]. In a subsequent study, the anti-apoptotic properties of EETs were further demonstrated in isolated myocardium from patients with cardiovascular disease, highlighting the observation that cellular mechanisms of EET-mediated cardioprotection in rodents also occur in the human heart [38].

3.3 Preservation of mitochondrial function and structure

Mitochondria provide the primary source of energy that fuels the contractile apparatus and act as key regulators of cell survival and death [45]. IR injury can cause significant mitochondrial damage resulting in cellular death and cardiac dysfunction [45]. Specifically ischemia causes the mitochondrial permeability transition pore (mPTP), a non-specific pore in the inner membrane of mitochondria, to open allowing free passage of molecules <1.5 kDa [46]. The accumulation of these molecules in the mitochondria due to prolonged pore opening results in mitochondrial dysfunction and integrity and eventually leads to irreversible cell death [46]. Signal transduction pathways, including a large proportion of those mentioned previously that are implicated in EET-mediated cardioprotection, converge onto the mPTP thereby promoting or suppressing its opening following cardiac injury [47]. In addition, the mitochondrial membrane potential ($\Delta\psi_m$), which reflects the electrochemical gradient important for ATP generation, influences mPTP opening: its depletion following cardiac injury enhances mPTP opening [46]. Agents that prevent these processes have been found to reduce infarct size and improve recovery of cardiac function [43]. Emerging evidence demonstrates that EETs possess potent protective effects directly limiting mitochondria damage. For example, hearts from αMHC-CYP2J2-Tr mice had limited mitochondrial swelling and fragmentation following IR compared to hearts from wild-type

littermate controls [48]. Cell culture experiments demonstrated that exogenous administration of EETs slowed the loss of $\Delta\psi_m$ and prevented opening of the mPTP following the induction of stress to cardiac cells; this was reversed by 14,15-EEZE [48, 49]. And recent data show that EET-mediated protection of mitochondria involves regulating an autophagic response, which shifts the cell pathway in starved cardiac cells from death via apoptosis or necrosis toward survival, representing a novel prosurvival mechanism in cardiac cells [50]. However, an important issue that remains unknown is how the EET protective signals reach the mitochondria and preserve its structure. One potential mechanism implicates caveolins (cardioprotective structural proteins) in the EET-mediated preservation of mitochondrial structural integrity following IR [51]. In particular, compared to untreated wild-type mice, plasma membrane and mitochondria isolated from the hearts of *Ephx2*$^{-/-}$ and EET-treated wild-type mice exhibited an attenuated IR-induced loss of caveolin-1 (Cav-1), but not caveolin-3 (Cav-3) isoform expression [51]. Altogether, these results underscore the important role of preserving mitochondrial function and architecture following IR injury.

Altogether, these preclinical findings in acute models of IR have made important contributions to the characterization of EET-mediated cardioprotection, confirming a direct protective effect of EETs on cardiomyocytes and offering mechanistic insight. It is worth reemphasizing that a structure-activity relationship exists with EETs based on the observations that 1) the endogenously formed EET regioisomers exert their biological effects at varying potencies, and 2) synthetic analogs of EETs agonize and antagonize their effects at varying degrees [52]. This is the main reason why, although it remains to be discovered, at least one EET-specific receptor (whether at the cell surface or intracellularly) is widely believed to exist [9, 53]. Thus, identification and characterization of an EET receptor would provide critical insight into the diverse biological effects of EETs, including cardioprotection, and drive future research and drug discovery.

4. Chronic EET effects following IR

Following the acute recovery phases of an AMI, the infarcted and inflamed myocardium chronically promotes LV remodeling, which manifests as scar tissue formation (fibrosis), LV dysfunction, and ultimately heart failure [54, 55]. Fibrosis following AMI leads to electrical conduction abnormalities, which predispose patients to ventricular arrhythmias and higher risk of sudden cardiac death [56]. Thus, this chronic maladaptive remodeling process is associated with worsened prognosis following AMI [57].

Chronic preclinical models of ischemic cardiomyopathy have recently been utilized to determine the impact of increasing EETs on longer term endpoints following AMI. In a mouse model of post-AMI heart failure, where the left anterior descending (LAD) coronary artery was occluded for 45 minutes followed by 3 weeks of reperfusion, administration of a sEH inhibitor reduced collagen deposition (fibrosis), reduced arrhythmia, and improved LV fractional shortening (systolic function) [58]. However, it is important to note that the sEH inhibitor was administered three days before the induction of AMI; thus it is unknown whether the observed attenuation of cardiac remodeling was derived independently of infarct size reduction and the other beneficial actions of EETs that occur acutely following IR. In a

clinical situation, a pharmacological agent indicated to reduce IR injury would most likely be administered during or after, and not prior to, the ischemic phase. Three recent studies have provided insight into this important issue. In one study, a sEH inhibitor was administered immediately following permanent LAD occlusion in rats; pharmacologic suppression of sEH attenuated LV ejection fraction (systolic function) independent of collagen deposition reduction in the infarct zone following 5 weeks of occlusion [59]. A second study by a separate group utilized a more chronic model of heart failure in rats (permanent LAD ligation for 50 days), but administered a sEH inhibitor at distinct time points after the initial phase of infarct healing: 8 days (42-day sEH inhibitor treatment) or 47 days (3-day inhibitor treatment) following LAD occlusion [60]. It was discovered that both regimens improved LV ejection fraction at 50 days; however, only long term treatment elicited an improvement in LV end-diastolic pressure (diastolic function) [60]. A third study in mice utilizing a model of IR injury demonstrated that sEH inhibition administered a week following AMI was still able to attenuate chronic collagen deposition measured three weeks later [61]. Collectively, these studies demonstrate that sEH inhibition improves maladaptive chronic ventricular remodeling independent of the aforementioned acute reductions in infarct size elicited by EETs. It is also important to note that these effects were not reported to be related to blood pressure reduction, suggesting that direct action on cardiomyocytes is a likely mechanism of cardioprotection. More rigorous investigation, including the use of other pharmacologic and genetic tools that promote or depress the effects of EETs, is warranted to further define the contribution of sEH and EETs to the pathogenesis and progression of ischemic cardiomyopathy.

5. Chronic EET effects in non-ischemic cardiomyopathy

Non-ischemic cardiomyopathy is caused by variety of factors unrelated to an AMI (i.e., longstanding hypertension) and is a source of devastating consequences including arrhythmia, left ventricular dysfunction, heart failure, and mortality [62]. Importantly, EETs have been found to be cardioprotective in rodent models of non-ischemic cardiomyopathy. Furthermore, in the majority of models, these protective effects were not reported to be dependent on blood pressure lowering.

Inhibition of sEH was found to reverse transverse aortic constriction (TAC)-induced cardiac hypertrophy [63]. Furthermore, angiotensin II, a potent driver of cardiac hypertrophy, upregulates sEH expression in cardiomyocytes and pharmacologic sEH inhibition attenuates angiotensin II-induced cardiac hypertrophy [64]. CYP2J overexpression also reversed decline in cardiac function from tumor necrosis factor alpha (TNF-α) administration to rats [65] and prevented the development of TAC-induced arrhythmias in mice [66]. These results are confirmed when sEH is modulated genetically, as *Ephx2*$^{-/-}$ mice were protected from cardiac dysfunction and arrhythmia following chronic high dose angiotensin II treatment or TAC banding [67]. In a study capitalizing on single nucleotide polymorphism differences in the putative *Ephx2* promoter region of two closely related strains of spontaneously hypertensive rats, spontaneously hypertensive heart failure rats were found to have increased transcript levels of *Ephx2* and lower 14,15-EET levels compared to spontaneously hypertensive rats that do not develop heart failure, further suggesting an important role for sEH-mediated EET hydrolysis in the development of heart failure unrelated to IR injury and

AMI [67]. In contrast to preclinical models of IR, EET-mediated cardioprotection in non-ischemic cardiomyopathy has not been demonstrated in non-rodent models and remains an important future direction for this line of investigation.

6. EET action in cardiac non-myocytes

In addition to cardiomyocytes, the heart is composed of fibroblasts, endothelial cells, and vascular smooth muscle cells [68]. It is clear that these other cell types are important in the production and action of EETs in the heart (Fig. 2A); however, the contribution of EETs that act on or are produced by these other cell types to the cardioprotection phenotype observed in preclinical studies is less certain.

6.1 Action of EETs derived from cardiac endothelial cells on myocardial cells

CYP epoxygenases and sEH are highly expressed in endothelial cells [69, 70]. Isolated hearts from transgenic mice with endothelial sEH or CYP2J2 overexpression (Tie2-sEH-Tr and Tie2-CYP2J2-Tr mice) did not alter the recovery of LVDP or infarct size following IR compared to wild-type mice, demonstrating that endothelial-derived EETs do not have a significant impact on acute myocardial recovery in this model of IR injury [71]. Intriguingly, isolated hearts from transgenic mice with endothelial CYP2C8 overexpression (Tie2-CYP2C8-Tr mice) had worsened LVDP and infarct size compared to hearts from wild-type mice [71]. This demonstrates that the specific CYP epoxygenase isoform catalyzing the formation of EETs in the endothelium appears to play an important role in cardiac function in mice [71]. Increased parallel production of reactive oxygen species (ROS) and linoleic acid-derived metabolites were found to be the cause of the enhanced IR injury in Tie2-CYP2C8-Tr mice [71]. Specifically, CYP2C8 overexpression catalyzed the formation of epoxyoctadecaenoic acids (EpOMEs, leukotoxin) from linoleic acid in endothelial cells, which are subsequently metabolized by sEH to dihydroxyoctadecaenoic acids (DHOMEs, leukotoxin diol) [71]. Enhanced endothelial DHOME, along with reactive oxygen species (ROS), formation mediated the cardiodepressive phenotype observed in Tie2-CYP2C8-Tr, but not Tie2-CYP2J2-Tr, mice [71]. It remains unclear whether similar CYP isoform specific effects occur in cardiomyocytes.

6.2 Action of EETs derived from cardiac endothelial cells on cardiac smooth muscle cells

Endothelial-derived EETs exert their vasodilatory action in coronary vessels through calcium-activated potassium channel (Kca)-dependent hyperpolarization of smooth muscle cells independent of prostaglandin or NO synthesis [72, 73]. Interestingly, this effect has been found to be greatest in smaller coronary arterioles, rather than larger epicardial coronary arteries [74]. Despite these findings, EET-mediated vasodilation in the setting of IR injury and its putative beneficial effect of aiding in the perfusion of oxygenated blood to ischemic regions of the heart remain unclear. Altogether, further work is necessary to validate the role of endothelial EETs in cardioprotection, especially in *in vivo* and chronic models of AMI.

6.3 Action of EETs in cardiac endothelial cells

Endothelial-derived EETs have well-established pro-angiogenic properties through a multitude of signaling pathways that are reviewed in great detail elsewhere [75]. Experimental studies demonstrate that angiogenesis is associated with cardioprotection in chronic phases following IR [76]. Few studies have investigated the role of EET-derived angiogenesis in cardioprotection following IR and as a result this topic remains poorly understood. One report revealed that inhibition of sEH promotes capillary tube formation (angiogenesis) in the isolated endothelial progenitor cells (EPCs) of post-AMI patients (compared to control subjects) through the EET–PPARγ pathway [77]. It is unknown if these effects occur with *in vivo* administration of a sEH inhibitor. Moreover, the EPCs were derived from whole blood and the effect of sEH inhibitor specifically on coronary vasculature formation was not evaluated [77]. Finally, it remains to be determined if these effects impact myocardial recovery following AMI. Consequently, further work is necessary to confirm and better understand these findings.

6.4 Action of EETs on inflammatory cells and cardiac fibroblasts

We mentioned earlier that inflammation promotes cardiac remodeling and fibrosis following myocardial IR injury. Although they are not a permanent cellular component of the myocardium, bone-marrow derived inflammatory cells such as monocytes and neutrophils drive this inflammatory process when they infiltrate the site of injury following IR [78]. Substantial evidence indicates that EETs, through inhibition of nuclear factor-kappaB (NF-κB) activation, attenuate inflammation in endothelial cells and monocytes [6] and mitigate macrophage/neutrophil infiltration in the vasculature [79]; these effects have not been extensively studied in the coronary vasculature following myocardial ischemic injury. Kompa et al. demonstrated that sEH inhibition impedes the infiltration of macrophages in the peri-infarct region of the myocardium in rats following permanent LAD ligation [59]. Intriguingly, sEH inhibition did not reduce macrophage infiltration in the infarct region of the myocardium [59]. Further investigation is warranted to fully characterize the contribution of inflammation reduction in EET-mediated cardioprotection. Inflammation precedes the development of chronic myocardial fibrosis during the following IR injury. Cardiac fibroblasts accelerate this maladaptive remodeling via secretion of growth factors and cytokines [68]. Inhibition of sEH has been found to directly block the proliferation, differentiation, migration, and secretion capacity of fibroblasts [59, 61]. More work is necessary to directly implicate EETs in sEH inhibition-mediated fibroblast suppression and to provide further mechanistic insight into this effect. Overall, further studies are necessary to determine the functional role of these cell-types in EET-mediated cardioprotection relative to cardiomyocytes.

7. Clinical studies investigating the role of EETs in the progression of CVD

Since pharmacological tools that directly and specifically manipulate EETs are currently not available for clinical use, we and others have relied on genetic and biomarker-driven observational studies to understand the role of the CYP epoxygenase-EET pathway in human CVD. Associations between the risk of developing a cardiovascular event and polymorphisms in genes coding for CYP2J2 [80–82], CYP2C8/9 [82, 83], and sEH

(*EPHX2)* [84–89] have been discovered. Studies evaluating genetic variation and risk of CVD development have been summarized in great detail elsewhere [10] and continue to be an active area of investigation [88, 89].

Inconsistencies in the strength of the associations between genetic variation in the CYP epoxygenase-EET pathway and CVD susceptibility have been reported across studies, suggesting that the relationship is likely complex and most profound in certain subsets of the population [10]. For instance, associations between *EPHX2*, *CYP2J2* and *CYP2C8* variants and CAD risk have often been most pronounced in cigarette smokers [81, 82, 84, 87]. Although the mechanism remains unclear, this suggests that the pathologic impact of genetic predisposition to alter CYP-derived EET levels may be greatest in the presence of underlying cardiovascular dysfunction. Indeed, modulation of CYP-derived EETs has minimal impact on basal cardiovascular function in preclinical models; whereas, the blood pressure lowering, anti-inflammatory, and cardioprotective effects are most substantial upon induction of a pathologic stimulus [6]. The relationship between genetic and metabolic variation in CYP epoxygenase-EET pathway genes and prognosis in patients with established CAD, however, has not been investigated and requires rigorous study.

Recently, we measured circulating eicosanoid metabolite concentrations in a cohort of patients with established and stable CAD and a corresponding population of healthy volunteers at low risk for CAD [90]. The 14,15-EET:DHET ratio in plasma (a biomarker of sEH metabolic function) was significantly greater in CAD patients relative to healthy volunteers suggesting that sEH metabolic function was suppressed in the presence of established CAD [90]. In concordance, plasma EET levels were also higher in CAD patients [90]. Given the aforementioned evidence demonstrating the cardioprotective effects of EETs in preclinical models of CVD, these findings allude to the possible presence of a compensatory increase in EET levels in the presence of established CAD. Interestingly, this observation is consistent with a prior study in which myocardial biopsies obtained patients who developed heart failure (defined as ejection fraction < 45%) secondary to CAD exhibited lower *EPHX2* mRNA expression compared to the biopsies obtained from control CAD patients without evidence of heart failure [67]. However, these preliminary observations must be interpreted with caution until further studies validate the observed differences in additional populations.

Despite the observed presence of lower sEH metabolic function and higher EET levels in this population of patients with established CAD, compared to healthy volunteer controls, substantial inter-individual variation in the 14,15-EET:DHET ratio and EET levels existed within the CAD population and the presence of obesity, advanced age, and cigarette smoking were the strongest predictors of low 14,15 EET:DHET ratios (higher sEH metabolic function) and low EETs [90, 91]. Consistent with the aforementioned preclinical evidence demonstrating the cardiovascular protective effects of EETs, lower 14,15-EET:DHET ratios (i.e., higher sEH metabolic function) and lower EET levels were significantly associated with pro-inflammatory phenotypes predictive of poor prognosis (higher circulating levels of the chemokine monocyte chemoattractant protein-1 and cellular adhesion molecules) [91]. Importantly, these associations were independent of clinical factors [91]. Taken together, these initial findings suggest that the subset of CAD patients

with enhanced sEH metabolic function and low EET levels may be predisposed to poorer prognosis and thus likely to derive therapeutic benefit from an intervention that promotes the biological effects of EETs. However, subsequent studies remain necessary to first determine the association between inter-individual variation in the CYP epoxygenase-EET pathway and prognosis (i.e., clinical outcomes rather than surrogate markers) in patients with existing CAD.

Importantly, evaluation of genetic and metabolic variation in the CYP epoxygenase-EET pathway, biomarkers of cardiovascular inflammation and cardiac remodeling, and prognosis in patients during and following the acute stage of an AMI has not been completed to date. Completion of such studies offers enormous potential to facilitate initial translation of the aforementioned growing body of preclinical evidence and guide the rational design of prospective, proof-of-concept clinical trials that aim to evaluate the cardioprotective effects of novel therapeutic strategies that promote the effects of EETs following an AMI.

8. Discussion: key considerations prior to initiation of proof-of-concept clinical trials

Despite major medical advances over the past four decades, there still exists a major need to develop cardioprotective therapies that reduce death and improve quality of life in AMI patients. Over the past decade, there have been numerous unsuccessful clinical trials involving novel AMI therapeutics that had shown initial promise in preclinical studies [4]. These failures underscore the complex pathophysiology of AMI and suggest that full preclinical elucidation into the cardioprotective effects of candidate agents is necessary to increase the probability for success in clinical trials. Past clinical trial failures were likely a consequence of being rushed through development before obtaining rigorous mechanistic insight in preclinical studies, and were therefore wrought by limitations in their design [4]. Specifically, important study design details such as timing and dose of intervention were determined based on theoretical evidence and not validated *a priori* in animal models of AMI [4]. Investigating the effects of a cardioprotective strategy across multiple experimental systems *in vitro*, *ex vivo*, and *in vivo* and ensuring that preclinical findings can be replicated across these experimental systems before human testing would allow for full mechanistic elucidation, facilitate a more accurate prediction of which drug candidates should be carried forward to humans, and ultimately improve clinical trial design. Significant initiatives have already begun to alleviate these major preclinical challenges in AMI therapy translation. For example, an NIH consortium of investigators called CAESAR (Consortium for preclinicAl assESsment of cARardioprotective therapies) has been set up to examine therapies in preclinical studies using the same rigorous standards that are used in clinical trials to ensure reproducibility and screen for truly effective therapeutic candidates [92].

Considering the thousands of interventions that have been reported to be cardioprotective in preclinical studies over the past few decades [92], it is not feasible for every promising intervention to be evaluated through this mechanism. Thus, a collective and collaborative effort between basic, clinical, and translational scientists using the same rigorous standards is needed to develop therapeutic strategies that promote the cardioprotective effects of EETs.

In addition to the aforementioned issues in the development of therapeutic strategies for AMI, further considerations must be kept in mind in order to facilitate specifically the translation of EET-promoting strategies into successful proof-of-concept phase I and II clinical trials.

8.1 Development of therapeutic strategies that promote EET action in humans

The successful development of this strategy for AMI is only as promising as the pharmacologic agents that modulate the CYP epoxygenase-EET pathway. The potential for clinical translation of exogenous EET administration is limited due to its short half-life and poor solubility. Synthetic EET analogs overcome this limitation. Historically, synthetic EET analogs conducive to *in vivo* dosing were not available [23]; however, in recent years, a novel generation of EET analogs have been developed. These improved agents are orally bioavailable and reach therapeutic levels in live animals as illustrated by their protective effects in *in vivo* rodent models of renal injury [93, 94]. Despite this steadfast improvement, lack of a known EET receptor continues to slow progress in this area. Discovery of the putative EET receptor(s) would further contribute to the field by elucidating structure-activity relationships and facilitating the design of agents with further improvements; efforts are ongoing.

Over the past decade, a variety of selective and potent sEH inhibitors have been optimized through structure-activity relationship techniques. The history of sEH inhibitor development is akin to that of EET analog development: compared to earlier members of the class, newer sEH inhibitors possess pharmacokinetic properties in animals predicted to be more favorable for oral human administration. Thus these agents are being actively developed and evaluated in both academic and pharmaceutical industry laboratories for a variety of indications and offer considerable promise as a therapeutic strategy for ischemic CVD [21, 95]. Thus, the sEH inhibitors and synthetic EET analogs are the most conducive therapeutic strategies poised for translation into humans in the near future. Investigators should prudently select new generation agents from these classes to evaluate their effects, especially in chronic and *in vivo* preclinical models of AMI where drugs ideal for chronic dosing are most important.

8.2 Potential unintended effects of increasing EET levels

EETs have a myriad of biological functions and therapeutically promoting these actions has the potential to cause unintended effects that should be considered. For instance, as mentioned earlier, EETs have potent vasodilatory effects. Although this could be beneficial in hypertensive patients, delayed restoration of blood pressure may have played a role in the increased mortality of *Ephx2*$^{-/-}$ mice observed in a model of cardiac arrest-induced hypotension followed by cardiopulmonary resuscitation (CPR) [96]. The precise cause of death, including the direct role of EETs, remains unknown, but these data may have important clinical implications since cardiac arrest and hypotensive shock are complications of AMI. Future preclinical studies are warranted to replicate these findings and implicate EETs as the causative mediator of this effect.

Despite their vasodilatory effects in the peripheral and coronary vasculature, EETs have vasoconstrictive effects in the pulmonary vasculature and may be important mediators in

pulmonary hypertension [97, 98]. *Ephx2*$^{-/-}$mice develop pulmonary vascular remodeling, right ventricular hypertrophy, and reduced exercise capacity (pulmonary hypertension) [99]. Interestingly, these phenotypes were not replicated in wild-type mice receiving chronic administration of a sEH inhibitor [99]. This suggests that the role of EETs and sEH in pulmonary hypertension is complex and further work is necessary to determine if EET-mediated pulmonary vasoconstriction is deleterious in the setting of an AMI or chronic cardiac remodeling.

Similar to Kca-mediated hyperpolarization of smooth muscle cells to induce vasodilation, EETs activate Kca-mediated hyperpolarization in platelets [100]. This leads to reduced platelet activation and prevents platelet adhesion to endothelial cells [100]. Such findings have been recapitulated *in vivo* in the skin muscle of hamsters [101] and in the cerebral arterioles of mice [102]. As agents that promote the EETs progress through development, we believe that the clinical relevance of this anti-platelet effect is enormous considering the well-established importance of long-term, anti-platelet therapies in patients suffering from an AMI [103, 104]. Of note, epoxydocosapentaenoic acids (EDPs) and epoxydocosapentaenoic acid (EEQs), which are CYP-dependent epoxy-derivatives of DHA and EPA, respectively (Fig. 1), are potent inhibitors of platelet aggregation [105]. Further preclinical studies are needed to grasp the potential additive/synergistic benefit of epoxyeicosanoid anti-platelet effects on top of their cardioprotective effects, specifically in the coronary arteries during IR injury.

EETs enhanced tumorigenesis and multiorgan metastasis in multiple mouse models of cancer [106, 107]. Specifically, these enhanced effects were observed in *Ephx2*−/−, Tie2-CYP2J2-Tr, and Tie2-CYP2C8-Tr mice; observed after the direct administration of EETs and sEH inhibitors; and mediated via the pro-angiogenic (VEGF-secreting) properties of EETs in the endothelium [106]. Though these effects have not been observed to occur in the absence of spontaneous tumor models, these findings raise a serious concern about the potential cancer causing ability of therapies that increase endogenous EET levels. Importantly, the pro-angiogenic effects of EETs also promote wound healing and tissue regeneration [108, 109], which may be beneficial in myocardial recovery following IR injury. Furthermore, EDPs inhibit tumor growth and metastasis through anti-angiogenic effects [110]; however, the impact of the synthetic EET analogs on these phenotypes has not been well characterized. More studies are clearly necessary to determine the role of CYP epoxygenase-EET and parallel metabolic pathways in the regulation of angiogenesis, especially in the setting of an AMI.

In addition to studying these biological effects of EETs for the purpose of understanding potential risks and benefits, this knowledge can also guide early drug design of EET-promoting agents. For example, a new series of sEH inhibitors have been modified to reduce their pro-angiogenic properties while maintaining potent and selective sEH inhibition [111]. Similarly, modification of synthetic EET analogs can alter their biological properties [112], (analogous to the aforementioned varying potencies across EET regioisomers). In fact, data suggests that EET-mediated cancer cell proliferation is regioisomer-specific [113]. Consequently, further research in this area is clearly needed to select the most promising compounds for further development and subsequent translation into clinical studies.

Furthermore, considering the complex course of pathological events in response to IR injury in the myocardium, optimizing the timing, dose, duration, and route of administration of EET-modulating therapeutics will be essential to maximize the cardioprotective benefits while minimizing the potential harmful effects of these strategies.

8.3 The use of clinically relevant models of AMI including assessing the impact of comorbidities

An additional reason for drug development failure is the evaluation of candidate agents in animal models that are not clinically-relevant and therefore poorly mimic the AMI patient population [92]. Indeed, the majority of preclinical AMI models in the literature involve young, healthy animals lacking the comorbid conditions that are typically associated with the AMI population in humans [4]. Importantly, efforts have begun to determine the impact of these conditions on EET-mediated cardioprotection. For instance, it is well known that aging, a risk factor highly prevalent in the AMI population, exacerbates myocardial IR injury [28]. Cardioprotection from cardiomyocyte-specific CYP2J2 overexpression in the isolated hearts of young αMHC-CYP2J2-Tr mice were lost in aged αMHC-CYP2J2-Tr hearts following global IR injury [114]. However, this loss was regained when aged αMHC-CYP2J2-Tr hearts were perfused with a sEH inhibitor, suggesting that pharmacological inhibition of sEH activity remains cardioprotective even in aged hearts [114]. Future studies in clinically-relevant AMI models are needed in order to determine if the cardioprotective effects of EETs are influenced by conditions such as aging, diabetes, and obesity.

8.4 Subsets of the AMI population may derive greater benefit from agents that promote the cardioprotective effects of EETs

Even after addressing all of the aforementioned considerations in the translation of EET-promoting therapeutics, the innate heterogeneity of the AMI population is an important barrier to the successful translation of AMI therapeutics [4] as novel treatments may not work in broad populations. As technology advances and we enter an era of personalized medicine, genetic and biochemical biomarkers offer considerable promise to identify which patients are defective in the specific pathway that the drug targets, are at increased risk of poor prognosis, and may derive the most benefit from that therapeutic intervention. Thus, selective administration of EET promoting therapy to a pre-identified population of ‘responders’ predisposed to low EET levels may increase the probability of clinical trial success. Notably, the *EPHX2* Lys55Arg polymorphism is a promising genetic biomarker that may help identify patients predisposed to low EET levels since variant carriers have enhanced sEH metabolic function and increased risk of incident CAD [84]. Moreover, direct measurement of EET levels has the potential to identify these patients. Indeed, higher throughput methods to quantify eicosanoids in a clinical setting have been found to be precise, accurate, and feasible [115]. Altogether, further work is necessary to determine 1) which biomarkers can best identify the subset of AMI patients with reduced EET levels and 2) if these patients have poorer prognosis before the initiation of clinical trials that test whether interventions are likely to be most effective in those subsets.

9. Summary/Conclusion

After a 1997 paper reported that EETs were cardioprotective in *ex vivo* and *in vitro* models [39], it took about 10 years before the first *in vivo* reports began to recapitulate these findings. Since then, much attention has been focused on the role of the CYP epoxygenase-EET pathway in AMI and the potential for therapeutic modulation of this pathway to improve outcomes in humans. In parallel, the improvement of experimental strategies to manipulate the pathway through pharmacologic and genetic approaches has allowed for great advances in the knowledge surrounding EET-mediated cardioprotection. Evidence for the beneficial effects of EETs has been replicated using multiple species, experimental heart disease models, and phenotypes relevant to cardioprotection, which underscores the great promise of this therapeutic strategy. Most of the data in the literature highlight the acute role of EETs in prosurvival pathway-mediated mitochondrial preservation in cardiomyocytes following AMI, but EETs have also been found to attenuate chronic cardiac remodeling and elicit protective effects in other cell types following AMI. It remains unknown whether EETs cause deleterious effects that could outweigh these cardioprotective benefits. Moreover, it is poorly understood if modulation of parallel epoxide and diol metabolites derived from other PUFAs impacts this benefit-risk ratio. Consequently, further work will be critical to move this line of investigation closer to clinical trials.

Ultimately, randomized controlled trials will be necessary to determine the benefits and risks of therapeutic strategies that promote the effects of EETs in patients experiencing an AMI. Before advancing promising agents that promote the effects of EETs into clinical trials, additional preclinical and human investigations are needed in order to lay an essential foundation for the rational design of these future prospective trials and select the agent, dosing strategy, and patient population most likely to circumvent clinical failure. A concerted effort to address these prerequisites will serve to improve the probability of translational success from a rapidly emerging body of evidence into a clinically applicable therapeutic strategy for patients with AMI.

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Non-standard abbreviations

$\Delta\psi_m$	mitochondrial membrane potential
AMI	acute myocardial infarction
CAD	coronary artery disease
Cav-1	caveolin-1
Cav-3	caveolin-3

CPR	cardiopulmonary resuscitation
DHETS	dihydroxyeicosatrienoic acids
DHOMEs	dihydroxyoctadecaenoic acids
eNOS	endothelial nitric oxide synthase
EDPs	epoxydocosapentaenoic acids
EETs	epoxyeicosatrienoic acids
EPCs	endothelial progenitor cells
EpOMEs	epoxyoctadecaenoic acids
IR	ischemia-reperfusion
Kca	calcium-activated potassium channel
LAD	left anterior descending
LV	left ventricular
LVDP	left ventricular developed pressure
mitoK$_{ATP}$	mitochondrial K_{ATP}
mPTP	mitochondrial permeability transition pore
NF-κB	nuclear factor-kappaB
NO	nitric oxide
p42/p44 MAPK	p42/p44 mitogen-activated protein kinase
PI3K	phosphatidylinositol-3 kinase
ROS	reactive oxygen species
sarcK$_{ATP}$	sarcolemma K_{ATP}
sEH	soluble epoxide hydrolase
TAC	transverse aortic constriction
TNF-α	tumor necrosis factor alpha

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Highlights

- EETs exert cardioprotective effects in animal models of acute myocardial infarction.
- Variation in the CYP epoxygenase-EET pathway is associated with CVD risk in humans.
- Agents that promote the effects of EETs are promising CVD therapies.
- Further research is necessary to facilitate the rational design of clinical trials.

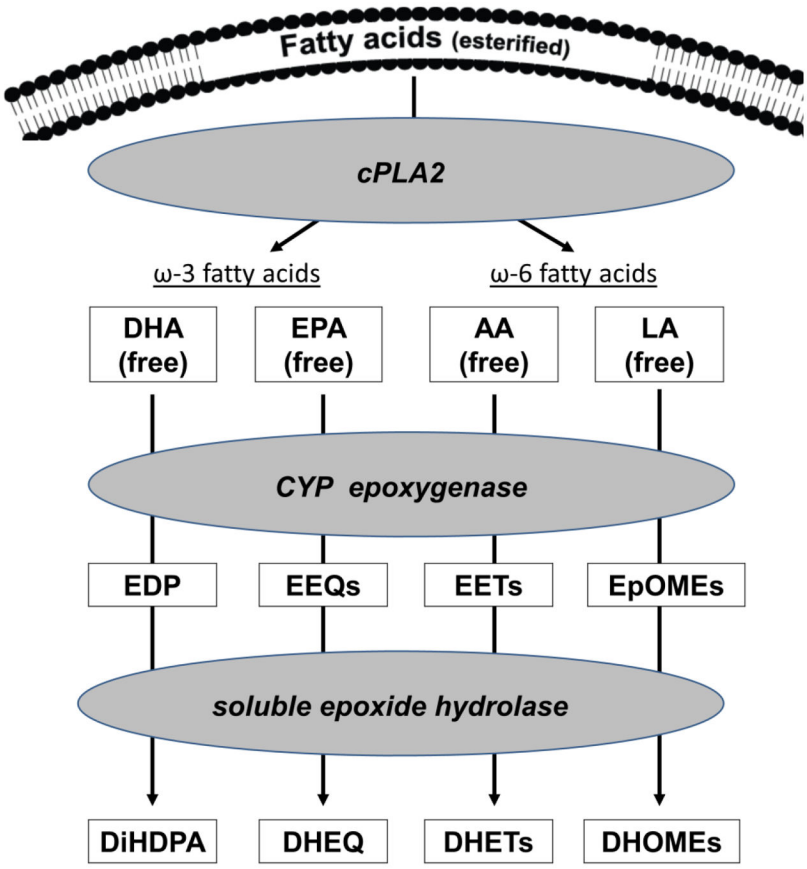

Fig. 1. Cytochrome P450 (CYP) epoxygenase-epoxyeicosatrienoic acid (EET) and parallel pathways

Through the activation of cytosolic phospholipase A2 (cPLA2) in cardiomyocytes following AMI, membrane-bound fatty acids are released into the cytosol and subsequently metabolized by CYP epoxygenases to form biologically active eicosanoids. The CYP2J and CYP2C epoxygenases produce four regioisomers of EETs from arachidonic acid (AA) that elicit various biological effects. These bioactive epoxyeicosanoids are extensively hydrolyzed by soluble epoxide hydrolase into the less biologically active dihydroxyeicosatrienoic acid (DHET) metabolites. DHA, docosahexaenoic acid; DHEQ, dihydroxy-eicosatetraenoic acid; DHOME, dihydroxyoctadecaenoic acid; DiHDPA, dihydroxy-docosapentaenoic acid; EDP, epoxydocosapentaenoic acid; EEQ, epoxyeicosatetraenoic acid; EPA, eicosapentaenoic acid; EpOME, epoxyoctadecaenoic acid; LA, linoleic acid

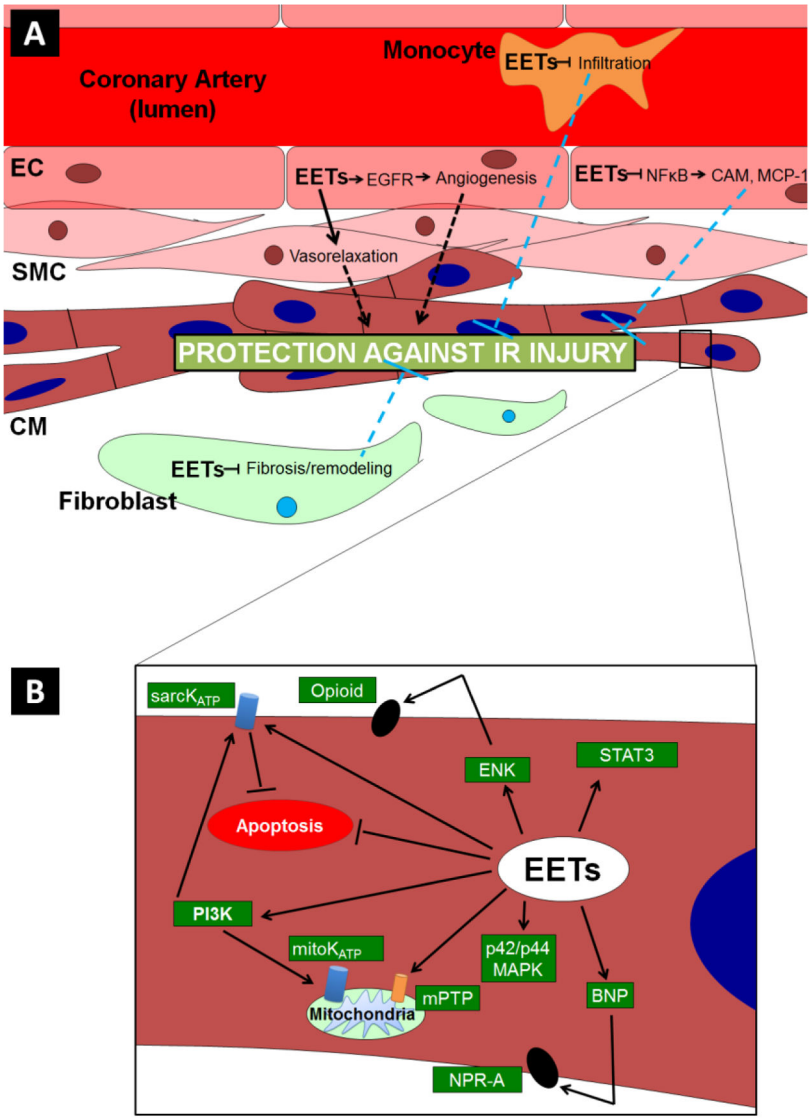

Fig. 2. Epoxyeicosatrienoic acid (EET) cardioprotection in the heart

A) EETs elicit action in cardiac endothelial cells (pro-angiogenic, anti-inflammatory), smooth muscle cells (vasodilatory), fibroblasts (anti-fibrotic), and inflammatory cells (anti-inflammatory). These effects may protect the myocardium following IR injury. B) EETs derived from cardiomyocytes (CMs) elicit direct cardioprotection of the myocardium during the acute phase following IR injury. They activate prosurvival signaling pathways, many of which converge onto mitochondria and promote its preservation. BNP, B-type natriuretic peptide; CAM, cell adhesion molecules; EC, endothelial cell; EGFR, epidermal growth factor receptor; ENK, enkephalin; IR, ischemia reperfusion; MCP-1, monocyte chemoattractant protein-1; mitoK$_{ATP}$, mitochondrial ATP-activated K+ channel; mPTP, mitochondrial permeability transition pore; NF-κB, nuclear factor-κB; NPR-A, natriuretic peptide receptor type-A; p42/p44 MAPK, p42/p44 mitogen-activated protein kinase; PI3K,

phosphoinositide 3-kinase; sarcKATP, sarcolemmal ATP-activated K+ channel; SMC, smooth muscle cell; STAT3, signal transducer and activator of transcription

Table 1

Mechanisms underlying direct cardioprotective effects of epoxyeicosatrienoic acids (EETs) on cardiomyocytes

EET action	Model	Mechanisms
Reduced myocardial cell death and apoptosis	Hypoxia-reoxygenation *(in vitro);* OGD/RGR (*in vitro*)	PI3K/Akt signaling [36, 38, 44] K_{ATP} channel signaling [37, 38] STAT3 signaling [40]
Improved recovery of left ventricular developed pressure	Global ischemia and reperfusion *(ex vivo)*	K_{ATP} channel signaling [30, 31, 37, 48] p42/p44 MAPK signaling [30] PI3K/Akt signaling [30, 31, 36, 41, 116] BNP/NPR-A signaling [41] Opioid signaling [42] Protein phosphatase 2A signaling [114] Reduced leukotoxin diol levels [114] Reduced oxidative stress [114] Preservation of caveolin-1 [51]
Reduced left ventricular infarct size	Global ischemia and reperfusion *(ex vivo);* Ischemia-reperfusion *(in vivo)*	PI3K/Akt signaling [31, 36] K_{ATP} channel signaling [26, 31–35, 37] STAT3 signaling [40] Opioid signaling [42] eNOS activation [35] Initial ROS formation [34] Inhibition of mPTP opening [35]
Reduced chronic left ventricular remodeling[a]	Ischemia-reperfusion *(in vivo)*	Increased eNOS : iNOS ratio [60] Reduced myocardial collagen deposition [58–61] Reduced cardiac fibroblast activation [59] Reduced inflammatory cytokines [58, 61] Reduced macrophage infiltration [59] Reduced oxidative stress [60]

[a] Chronic ischemia-reperfusion involved reperfusion periods of at least 2 weeks following ischemia

Akt, protein kinase B; BNP, B-type natriuretic peptide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; KATP, ATP-activated K^+ channel; mPTP, mitochondrial permeability transition pore; NPR-A, natriuretic peptide receptor type-A; OGD, oxygen and glucose deprivation; p42/p44 MAPK, p42/p44 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; RGR, reoxygenation and glucose repletion; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3