Role of B-type natriuretic peptide in epoxyeicosatrienoic acid-mediated improved post-ischaemic recovery of heart contractile function

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Aims This study examined the functional role of B-type natriuretic peptide (BNP) in epoxyeicosatrienoic acid (EET)-mediated cardioprotection in mice with targeted disruption of the sEH or Ephx2 gene (sEH null).

Methods and results Isolated mouse hearts were perfused in the Langendorff mode and subjected to global no-flow ischaemia followed by reperfusion. Hearts were analysed for recovery of left ventricular developed pressure (L VDP), mRNA levels, and protein expression. Naïve hearts from sEH null mice had similar expression of preproBNP (Nppb) mRNA compared with wild-type (WT) hearts. However, significant increases in Nppb mRNA and BNP protein expression occurred during post-ischaemic reperfusion and correlated with improved post-ischaemic recovery of L VDP. Perfusion with the putative EET receptor antagonist 14,15-epoxyeicosa-5(Z)-enoic acid prior to ischaemia reduced the preproBNP mRNA in sEH null hearts. Inhibitor studies demonstrated that perfusion with the natriuretic peptide receptor type-A (NPR-A) antagonist, A71915, limited the improved recovery in recombinant full-length mouse BNP (rBNP)- and 11,12-EET-perfused hearts as well as in sEH null mice. Increased expression of phosphorylated protein kinase C ε and Akt were found in WT hearts perfused with either 11,12-EET or rBNP, while mitochondrial glycogen synthesize kinase-3β was significantly lower in the same samples. Furthermore, treatment with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin abolished improved L VDP recovery in 11,12-EET-treated hearts but not did significantly inhibit recovery of rBNP-treated hearts.

Conclusion Taken together, these data indicate that EET-mediated cardioprotection involves BNP and PI3K signalling events.

KEYWORDS
B-type natriuretic peptide; Epoxyeicosatrienoic acid; Ischaemia-reperfusion; GSK-3β

1. Introduction

Arachidonic acid (AA), an essential polyunsaturated fatty acid found esterified to membrane phospholipids, can be metabolized by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) epoxygenases to numerous products collectively termed eicosanoids, which have important cellular functions.1,2 Ischaemic events activate cytosolic phospholipase A2, which will release AA from stores of glycerophospholipids.3 AA can then be metabolized by various CYPs to four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs), all of which are biologically active.2 Modulation of EET levels can occur via re-incorporation into phospholipid membranes or by β-oxidation to smaller reactive epoxides;4,5 however, the predominant pathway occurs through metabolism to inactive vicinal diols compounds by epoxide hydrolases.1,6 Two major epoxide hydrolases are found in mammalian tissues—microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH or Ephx2).5

Accumulating evidence indicates that EETs have important functional roles in ischaemia-reperfusion injury. They activate various stress response systems, such as the phosphoinoside 3-kinase (PI3K) pathway; enhance membrane ion channel activity, such as sarcolemmal (sarcKATP) and mitochondrial (mitoKATP) ATP-sensitive K+ channels; and improve post-ischaemic recovery of left ventricular function.7-12 Evidence indicates that EET-mediated cardioprotective signals target glycogen synthesize kinase-3β (GSK-3β), a key protein involved in cellular function and

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signalling essential to cardioprotection.\textsuperscript{13,14} Regulated via phosphorylation, GSK-3\(\beta\) acts as a central convergence point targeting upstream signalling to mitochondria.\textsuperscript{13,15} Published reports indicate that multiple protective pathways converge upon GSK-3\(\beta\) prior to effecting mitochondrial function.\textsuperscript{7,13,16} Alteration in the production and/or elimination of EETs may affect steady-state cellular levels of these bioactive eicosanoids in vivo and could potentially influence cardiac function.

B-type or brain natriuretic peptide (BNP) is becoming a valuable biomarker for cardiovascular disease that bears diagnostic, prognostic, and therapeutic importance in congestive heart failure, arrhythmias, and acute myocardial infarction.\textsuperscript{17,18} Evidence indicates that BNP can attenuate ischaemic-reperfusion injury in animal models but the underlying mechanism is unknown.\textsuperscript{19,20} Isolated perfused rat heart studies suggest that increased BNP expression at baseline and release of peptide in the coronary effluent during reperfusion are attributed to wall stretch and acute ischaemic injury.\textsuperscript{21,22} Cardioprotective effects of BNP correlate with elevated cGMP and NO levels, which can be abolished by inhibiting the mitoK\(_{ATP}\) channel.\textsuperscript{20,21} The specific involvement of BNP and mitoK\(_{ATP}\) opening in cardioprotection is largely unknown. The anti-ischaemic profile of natriuretic peptides and correlation to mitoK\(_{ATP}\) suggest that endogenous BNP may be an attractive target for cardioprotection and may warrant further investigation.

Recently, we reported that mice with the targeted disruption of the Ephx2 gene (sEH null), originating from Darryl Zeldin (NIH/NEIH) and backcrossed onto a C57BL6 genetic background for more than seven generations, is maintained at the University of Alberta. C57BL6 mice and New Zealand White rabbit were purchased from Charles River Laboratories (Pointe Claire, PQ). All experiments used male and female mice aged 3–5 months, weighing 20–34 g and were treated in accordance with the guidelines of Health Science Laboratory Animal Services (HSLAS), University of Alberta. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.3 Animals

A colony of mice with targeted disruption of the Ephx2 gene (sEH null), originating from Darryl Zeldin (NIH/NEIH) and backcrossed onto a C57BL6 genetic background for more than seven generations, is maintained at the University of Alberta. C57BL6 mice and New Zealand White rabbit were purchased from Charles River Laboratories (Pointe Claire, PQ). All experiments used male and female mice aged 3–5 months, weighing 20–34 g and were treated in accordance with the guidelines of Health Science Laboratory Animal Services (HSLAS), University of Alberta. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.4 Isolated heart perfusions

Hearts were perfused in the Langendorff mode as previously published.\textsuperscript{14,27} Briefly, hearts were perfused with Krebs–Henseleit buffer for 40 min of baseline and subjected to 20 or 30 min of global no-flow ischaemia followed by 40 min of reperfusion. For some experiments, hearts were perfused with rBNP (10 nmol/L), 11,12-EEZE (1 \(\mu\)mol/L), NPR-A antagonist A71915 (50–100 nmol/L) (Bachem, USA), PI3K inhibitor wortmannin (200 nmol/L) (Sigma-Aldrich, Oakville, ON) or the putative EET receptor antagonist 14,15-EEZE (1 \(\mu\)mol/L) throughout 40 min of reperfusion. The percentage of left ventricular developed pressure (%L VDP) at 40 min of reperfusion (R40), when compared with baseline LVDp, was taken as a marker for recovery of contractile function. After 40 min of reperfusion, hearts were immediately frozen and stored below \(-20^\circ\)C.

### 2.5 Gene expression

Quantitative real-time PCR (qPCR) analysis of natriuretic peptide precursor type B (NM_008726) expression was performed in hearts from naïve mice and isolated perfused experiments. Some isolated hearts were treated with the PI3K inhibitor, LY294002 (5 \(\mu\)mol/L) or the putative EET receptor antagonist 14,15-EEZE (100 nmol/L) prior to ischaemia. Total RNA was isolated using an RNeasy Midi kit (Qiagen, Valencia, CA, USA) and concentrated using a Microcon YM-30 column (Millipore, Billerica, MA, USA). A formaldehyde agarose gel containing ethidium bromide was used to assess the quality of the RNA. mRNA from individual hearts were treated with DNaseI and then 1 \(\mu\)g was used to prepare cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). cDNA levels were detected using qPCR with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Each individual sample was normalized to the RNA expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within the same heart. Fold expression change was determined by quantitation of cDNA from target (perfused) samples relative to a calibrator sample (naïve) of the same genotype. To determine this normalized value, \(2^{-\Delta\Delta Ct}\) values were compared between target and calibrator samples, where \(\Delta Ct = \text{target gene} - \text{(cycling}
expression of Nppb mRNA in sEH null or in WT hearts. PI3K inhibitor, did not affect the increased post-ischaemic mRNA levels in sEH null hearts but had no effect on WT hearts (Figure 1A). Perfusion of hearts with LY294002, a PI3K inhibitor, did not affect the increased post-ischaemic expression of Nppb mRNA in sEH null or in WT hearts (Figure 1A).

While immunoblotting with the anti-mouse BNP antibody demonstrated no significant differences in BNP protein expression following aerobic perfusion (Figure 1B), a significant increase in post-ischaemic BNP protein expression was observed in sEH null hearts compared to WT hearts (Figure 1C). In addition, significantly higher expression of BNP was observed in post-ischaemic WT hearts perfused with exogenous 11,12-EET (Figure 1C). Densitometric analysis revealed that BNP expression in sEH null mice hearts was four times higher than in WT hearts while exogenous 11,12-EET-treated WT hearts had two times higher BNP expression than vehicle-treated WT hearts (Figure 1C). EET-mediated increased expression was attenuated by co-treatment with 14,15-EEZE (Supplementary material online, Figure S3).

Figure 1 PreproBNP mRNA and protein expression. (A) qPCR data showing relative expression of Nppb to GAPDH mRNA in non-perfused (naïve) wild-type and sEH null mouse hearts, during 20 min aerobic baseline perfusion (base) and at 40 min post-ischaemic reperfusion (reperfusion). (B) Representative immunoblots showing expression of BNP in WT, sEH null, and 11,12-EET- (1 μmol/L) treated hearts during aerobic baseline. (C) Representative immunoblots showing densitometry showing relative abundance of BNP to GAPDH in WT, sEH null, and 11,12-EET- (1 μmol/L) treated hearts at 40 min reperfusion following global ischaemia. Values represent mean ± SEM (n = 6 per group). *P < 0.05 vs. WT.

3.2 NPR-A signalling in EET and rBNP post-ischaemic contractile function

Consistent with our previous observations, sEH null and WT mice had normal baseline contractile function as measured by LVPD. Furthermore, sEH null hearts had improved post-ischaemic LVPD recovery following 30 min of global ischaemia and 40 min reperfusion compared with WT hearts (35.6% vs. 17.2%, respectively, P < 0.05) (Figure 2A). Likewise, treatment of WT hearts with 11,12-EET improved...
Recent evidence suggests a role for BNP in the recovery of post-ischaemic cardiac function.\(^{19,30,31}\) The increased BNP expression in sEH null and 11,12-EET-treated hearts suggested the hypothesis that BNP may be a mediator of the cardioprotective effects of EETs. To examine the role of BNP, we first perfused WT mouse hearts with exogenous rBNP (10 nmol/L) to study the effect of our recombinant protein on post-ischaemic contractile function. Hearts treated with exogenous rBNP did not demonstrate significant changes to baseline LVDP (data not shown); however, these BNP-treated hearts showed a significant increase in post-ischaemic functional recovery when compared with vehicle-treated control hearts (62% vs. 23%, respectively, \(P < 0.05\)) (Figure 2B). Perfusion with the natriuretic peptide receptor-A (NPR-A) antagonist A71915 attenuated the improved post-ischaemic functional recovery in WT hearts perfused with rBNP (Figure 2B). To further assess the role of BNP in EET-mediated cardioprotection, WT hearts were perfused with 11,12-EET in the presence or absence of A71915. Perfusion with A71915 during ischaemia and reperfusion significantly inhibited, but did not completely reverse, the improved post-ischaemic recovery observed in 11,12-EET-treated hearts (Figure 2B). Moreover, a significant reduction in post-ischaemic functional recovery of sEH null hearts was observed when perfused with A71915 (Figure 2A). In contrast, there was no effect of A71915 treatment on WT hearts (Figure 2A). Together, these data suggest that 11,12-EET-mediated cardioprotection is dependent upon the NPR-A receptor.

To further assess downstream targets of the NPR-A cascade, we examined the expression of p\(^{\text{Ser729}}\)PKC\(_b\) in mitochondrial and cytosolic fractions. In 11,12-EET-perfused hearts, significant increases in the phosphorylation of PKC\(_b\) were observed in mitochondrial fraction while a clear trend towards increase in cytosolic fractions were observed at 40 min of reperfusion (Figure 3A and C). Similar results were obtained from rBNP-perfused hearts with significant increases in mitochondrial and cytosolic p\(^{\text{Ser729}}\)PKC\(_b\) levels (Figure 3B and D). Evidence suggests that PKC\(_b\)-mediated cardioprotection involves increased phosphorylation and subsequent inactivation of GSK-3\(\beta\).\(^{13}\) A significant decrease in mitochondrial total GSK-3\(\beta\) expression was observed in 11,12-EET and rBNP-treated hearts when compared with vehicle-treated controls at R40 (Figure 4A). Conversely, higher expression levels of phosphorylated GSK-3\(\beta\) and total GSK-3\(\beta\) were observed in cytosolic fractions from hearts perfused with 11,12-EET or rBNP (Figure 4B). Together these data indicate that 11,12-EET and BNP trigger phosphorylation of PKC\(_b\) and this event is associated with phosphorylation and inactivation of GSK-3\(\beta\).

### 3.3 Role of PI3K pathway in EET- and BNP-mediated cardioprotection

As previously documented, EET-mediated cardioprotection involves activation of a PI3K pathway while BNP causes activation of a NPR-A/cGMP/PKG pathway.\(^{14,19,30}\) Partial inhibition of EET-mediated cardioprotection by A71915 (Figure 2B) suggests that EETs may work through multiple pathways. Therefore, we investigated the contribution of PI3K and NPR-A in the EET–BNP-mediated cardioprotection. Hearts from WT mice were perfused with 11,12-EET or rBNP in the presence or absence of the PI3K inhibitor wortmannin. Post-ischaemic functional recovery was significantly inhibited in hearts co-perfused with 11,12-EET and wortmannin but did not return to vehicle control levels (Figure 5A). Interestingly, wortmannin also resulted in a partial inhibition of post-ischaemic functional recovery in hearts co-perfused with rBNP, although these changes were not statistically significant (Figure 5A). To further assess downstream targets in the PI3K cascade, we examined the phosphorylation status of Akt following ischaemia-reperfusion. Expression levels of p\(^{\text{Ser473}}\)Akt were significantly higher in 11,12-EET- and rBNP-treated hearts when compared with vehicle-treated controls at R40 (Figure 5B and C). Conversely, the ratio of p\(^{\text{Ser473}}\)Akt to total Akt expression was significantly decreased in WT hearts perfused with both rBNP and A71915 (Figure 5C). These data suggest that EET-mediated signalling down the PI3K pathway does not occur solely via the NPR-A receptor.
4. Discussion

Since the first report of the cardioprotective effects of CYP epoxygenase metabolites of arachidonic acid against ischaemia-reperfusion injury by Wu et al., there has been considerable interest in investigating this novel protective mechanism. However, important questions remain unanswered regarding the molecular and cellular mechanisms responsible for this protection. Evidence suggests that cardioprotective signalling of EETs involves activation of PI3K/Akt pathway which further prevents ischaemic injury by reducing mitochondrial damage through activation of mitoKATP channels. Herein, we demonstrate for the first time, that targeted disruption of the Ephx2 gene and treatment with exogenous 11,12-EET leads to increased BNP expression following ischaemia-reperfusion resulting in improved functional recovery. Moreover, our data suggest a critical role of BNP and GSK-3β in mediating the cardioprotective effect of EETs. Thus, EET-mediated cardioprotection involves two pathways, PI3K/Akt and BNP-PKC1-mediated signalling, which converge on the mitochondria. Taken together, these data suggest that EETs have important intracellular protective effects in the ischaemic heart that limits functional damage attributed to ischaemia-reperfusion injury.

BNP is a cardiac hormone predominantly found in ventricles of heart. Considered an immediate-early gene, it can be both rapidly synthesized and released within 1 h in response to stimuli like stretch, volume, pressure overload and ischaemia. Basal expression levels of Nppb mRNA were found to be similar in naïve sEH null and WT hearts, indicating targeted disruption of Ephx2 gene does not alter BNP under normal conditions. Studies in isolated rat hearts demonstrated that increased Nppb mRNA expression was observed during aerobic perfusion, reflecting a stretch-induced activation. We observed an increased Nppb mRNA levels during aerobic perfusion, which was slightly higher in sEH null hearts compared with WT hearts. This early increased expression did not translate into elevated BNP protein levels. However, the significant increases in Nppb mRNA levels observed in sEH null hearts following ischaemia-reperfusion correlated with large increases in BNP protein levels in sEH null hearts and WT mice perfused with EETs. Consistent with our previous results from sEH null
mice, cardiac effects of sEH disruption occurred only after ischaemia, presumably owing to enhanced release of EETs from phospholipid stores. A role for EETs in the induction of BNP is suggested by our observation that the putative EET receptor antagonist 14,15-EtOEt attenuates the increase in Nppb mRNA in sEH null mice.

Apart from mechanical stimulus, BNP gene expression can be induced in response to various factors such as endothelin-1, β-adrenergic stimulation, and proinflammatory cytokines, like interleukin-1β. Interleukin-1β induces BNP gene expression, partially through a Ras, Rac, and p38 kinase pathway, where p38 kinase acts on the MCAT element at position-97 of human BNP gene promoter. Alternatively, stabilization of Nppb mRNA via an MAPK and PKC-dependent mechanism results in increased levels as demonstrated in neonatal rat cardiomyocytes. While the mechanism for EET-mediated activation of BNP is not known, the observation that LY294002 did not affect increased Nppb mRNA in sEH null hearts suggests that the PI3K pathway is not involved. Our results demonstrate both stretch and ischaemia-reperfusion injury increase Nppb mRNA expression in WT hearts, which was markedly enhanced in sEH null hearts. Importantly, increased Nppb mRNA correlated with elevated BNP protein expression in hearts of both sEH null and WT mice perfused with EETs following ischaemia-reperfusion. These results provide further evidence for the cardioprotective effects of EETs and suggest they act as a novel regulator of BNP release.

Following ischaemia, rapid gene expression and de novo peptide synthesis occurs which results in increased plasma levels of BNP in acute myocardial infarction patients. BNP plasma concentrations are biomarkers for heart disease providing a valuable diagnostic tool for cardiac patients. However, the role of BNP in the pathophysiology of ischaemia-reperfusion injury and whether the heart is a target remains to be elucidated. BNP can exert autocrine or paracrine effects by binding with natriuretic peptide receptor type-A (NPR-A), which activates particulate guanylyl cyclase (pGC), thereby increasing formation of cGMP leading to activation of PKG. PKG can then activate PKC, particularly PKCe, which will translocate to mitochondria interacting with various mitochondrial membrane proteins, such as mitoKATP channels. Translocation of PKCe to mitochondria and activation of mitoKATP channels are believed to protect the myocardium from ischaemia-reperfusion injury, with a net effect of protecting mitochondrial function. In the present study, we observed increased levels of PKCe in mitochondrial fractions of hearts perfused with 11,12-EET or rBNP, which correlated with improved post-ischaemic functional recovery. Furthermore, administration of NPR-A antagonist, A71915, attenuated the improved post-ischaemic recovery of sEH null hearts and hearts perfused with rBNP peptide, but only partial inhibition was observed with exogenous EETs. Taken together, our experiments suggest that EET-mediated cardioprotection, at least in part, involves NPR-A activation of intracellular pathways that target the mitochondria.

GSK-3β is a constitutively active protein which is an important regulator of cellular function and a key enzyme in response to myocardial ischaemia-reperfusion injury. Inhibition of GSK-3β, either by increased phosphorylation or pharmacological inactivation, is known to reduce cell death caused by ischaemia-reperfusion injury. Increased expression of GSK-3β in mitochondria following ischaemia-reperfusion has been reported in cardiomyocytes and isolated heart experiments. The significance and mechanism of GSK-3β translocation has not been determined but evidence suggests a role for PI3K and PKC kinases. In addition, the specific effect of increased mitochondrial GSK-3β is not completely known but potentially involves modulation of mitochondrial permeability transition (mPTP). Recent work by Das and coworkers reports that inhibition of GSK activity induces dephosphorylation of VDAC, which subsequently preserves ATP levels, prevents calcium overloading, and oxidant stress independent of mPTP opening. Phosphorylation of GSK-3β by upstream kinases like Akt and PKC inactivate GSK-3β preventing myocardial damage from ischaemia-reperfusion injury. Previously, we demonstrated that cardioprotection in sEH null mice involves activation of PI3K followed by phosphorylation of GSK-3β. Interestingly, our current results demonstrate a
Figure 5  PI3K activation in EET- and BNP-mediated cardioprotection. (A) LVDP recovery at 40 min of reperfusion as percentage of baseline. Hearts treated with exogenous rBNP (10 nmol/L) or 11,12-EET (1 μmol/L) in presence and absence of PI3K antagonist (wortmannin) (200 nmol/L). Values represent mean ± SEM (n = 5–7 per group). *P < 0.05 vs. vehicle control; **P < 0.05 vs. treated with 11,12-EET. (B) Representative immunoblot and densitometry demonstrating expression of pser473Akt to total Akt in control, 11,12-EET-treated, or 11,12-EET- and A71915-treated C57BL6 mice hearts. Values represent mean ± SEM (n = 3 per group). *P < 0.05 vs. vehicle control. (C) Immunoblot and densitometry demonstrating expression of pser473Akt to total Akt expression in control, rBNP-treated, or rBNP- and A71915-treated hearts. Values represent mean ± SEM (n = 6 per group). *P < 0.05 vs. vehicle control.

Figure 6  Schematic diagram showing pathways of EET-mediated improved left ventricular function. We propose that the enhanced post-ischaemic functional recovery attributed to EETs is mediated through two different signalling pathways—PI3K/Akt and BNP/NPR-A signalling. First, EETs can activate the PI3K/Akt pathway which will inactivate GSK-3β. Or second, EETs can activate the BNP/NPR-A pathway, which in turn can either activate Akt independent of PI3K, or activate PKCe.
significant reduction in active GSK-3β in mitochondrial fractions of both 11,12-EET and rBNP-treated hearts when compared with controls. In addition, we observe increased levels of active Akt (pser473Akt) and inactive GSK-3β in cytosolic fractions, we predict that EETs and BNP either prevent the translocation of GSK-3β to the mitochondria or enhance the translocation from the mitochondria. In addition, our data suggest that BNP-mediated effects on GSK-3β do not involve PI3K/Akt pathway but activation of Akt via the NPR-A receptor. In contrast, EET-mediated events involve both PI3K/Akt and enhanced release of BNP. These data are consistent with evidence suggesting that inhibition of GSK-3β is a key component in integrating protective stimuli with mitochondria. However, the relative contribution of each pathway (PI3K/Akt or NPR-A) to the regulation of GSK-3β is unknown and requires further investigation.

We propose that enhanced post-ischaemic functional recovery attributed to EETs is mediated through two different signalling pathways—PI3K/Akt and BNP/NPR-A signalling. Figure 6 shows a schematic of the proposed mechanisms, whereby EETs can trigger the release of BNP to act in an autocrine manner or activate the PI3K pathway. Together the data in the current manuscript provide further evidence for the beneficial effects of EETs and suggest a novel mechanism for BNP in cardioprotection.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: None declared.

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