The contribution of endothelial nitric oxide synthase to early ischaemic preconditioning: the lowering of the preconditioning threshold. An investigation in eNOS knockout mice

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Abstract
Nitric oxide (NO) is implicated in triggering and mediating delayed preconditioning. However, the role of NO and endothelial nitric oxide synthase (eNOS) in early ischaemic preconditioning is controversial. To investigate the role of eNOS in this paradigm further, the response of hearts isolated from either eNOS knockout (KO) or eNOS wild type (WT) mice to various ischaemic-preconditioning stimuli were studied. To determine whether eNOS is pivotal to early ischaemic preconditioning, hearts from both groups were subjected to a robust ischaemic-preconditioning stimulus consisting of four cycles of 5 min ischaemia and reperfusion (4PC) prior to an injurious ischaemia/reperfusion insult. Both WT and KO animals demonstrated significant attenuation of infarct size from 34.91±2.14 to 21.32±2.53% and from 30.92±1.87 to 22.60±2.98%, respectively. Whilst eNOS/NO appears not to be pivotal to early ischaemic preconditioning, NO can nonetheless mediate protection in both WT and KO hearts, as the NO donor, S-nitroso N-acetyl penicillamine (2 μM), was seen to mimic preconditioning protection. Therefore, it appears that the targets for NO mediated protection exist in both WT and KO hearts. Reducing the number of preconditioning cycles from four to three to two continued to result in significant attenuation of infarct size in WT hearts (26.30±2.86 and 26.70±1.95%). However, early ischaemic preconditioning failed in the KO hearts with any preconditioning stimulus less robust than the four cycle protocol used in the initial experiments (3PC 32.51±3.04%, 2PC 28.64±2.55%). Therefore, whilst eNOS is not essential for robust early ischaemic preconditioning, NO can be shown to be cardioprotective in its own right, and, moreover, eNOS and thus nitric oxide synthesis may contribute to early ischaemic preconditioning by lowering the ischaemic threshold for protection.

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1. Introduction
Preconditioning confers two phases of resistance to myocardial infarction. The first phase, early preconditioning, has near immediate onset and lasts for up to 3 h [1]. The second phase, delayed preconditioning or 'second window of protection' (SWOP), appears 24 h later and has a duration of up to 3 days [2]. Nitric oxide (NO) has been linked to both the triggering [3] and mediation of delayed preconditioning [4,5]. Given the observations linking constitutively active nitric oxide synthases (NOS) to the trigger phase of delayed preconditioning, the lack of evidence for the role of nitric oxide in early ischaemic preconditioning is surprising [6–10], although NO synthesis has been linked to early preconditioning triggered by over-drive pacing [11].

Nitric oxide is synthesised by the catalysis of the substrate L-arginine by one or more of the three isoforms of NOS found in the myocardium: neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). Two of

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these NOS isoforms, eNOS and nNOS, are constitutively expressed and regulated by cytosolic calcium concentration and the presence of co-factors such as tetrahydrobiopterin (BH₄), magnesium, and NADPH [12]. In contrast, iNOS activity is independent of calcium, being to a large extent protein expression dependent [13]. In naive unstressed myocardium, iNOS protein expression is detectable at low levels; its presence is not thought to contribute significantly to early ischaemic preconditioning [14], although through triggered up-regulation is found to contribute to delayed ischaemic preconditioning [14].

Transient ischaemia used to trigger early ischaemic preconditioning results in the release of a number of metabolites and neurohumeral agents that trigger the preconditioning response. In characterising these ‘preconditioning-mimetics’, it has been noted that the dose response relationship between agonist and myocardial infarct sparing is not linear, but demonstrates a dose–response threshold. If the duration of transient ischaemia or the concentration of the preconditioning-mimetic is inadequate, no resistance to lethal ischaemic injury is observed. By progressively incrementing this preconditioning stimulus, a threshold is breached and the preconditioning response triggered. This ‘threshold’ hypothesis, as proposed by Downey’s group, is supported by studies in both rabbit [15] and man [16], whereby sub-threshold ischaemia can trigger preconditioning in the presence of an inhibitor of bradykinin breakdown. The summation of the increased bradykinin in the extracellular milieu with the sub-threshold ischaemia is sufficient to trigger preconditioning, and is blocked by the bradykinin B₂ receptor antagonist, HOE140.

Interestingly, many of the triggers of preconditioning also regulate eNOS activity, bradykinin being a prime example. Bradykinin is capable of modulating eNOS activity by displacement of the enzyme from inhibitory binding sites found on the sarcolemma [17] and the B₂ receptor itself [18]. Moreover, bradykinin can up-regulate eNOS activity through the phosphorylation of the putative up-regulatory serine residue 1177 [19–22], an event purported to increase the activity of eNOS by 40-fold. This latter regulatory step is catalysed by phosphatidylinositol 3 kinase (PI3 kinase) and cyclic adenosine monophosphate (cAMP); both enzymes have been demonstrated to be activated in the context of transient ischaemia associated with early ischaemic preconditioning [23,24].

Therefore, there is good evidence to suggest that eNOS activity should be up-regulated in the context of early ischaemic preconditioning. Given the quantity and distribution of constitutive expression of eNOS in the myocardium relative to other isoforms of NOS [25], it is attractive to hypothesise that nitric oxide generated from eNOS may play an important role in the mediation of early ischaemic preconditioning.

Therefore, we hypothesised that either eNOS (i) plays a pivotal role in the induction and mediation of early preconditioning, or (ii) contributes to lower the preconditioning threshold. To test these hypotheses, mice with either a targeted disruption of the eNOS gene or their age/mass matched wild types were compared with respect to their response to varying intensities of an early ischaemic preconditioning regimen.

2. Methods

2.1. Animals

Adult, mixed sex mice (22–28 g) were used in this investigation, either with targeted disruption of the eNOS gene (KO) (as described by Huang et al. [26]) or wild types (WT) of comparable genetic background (B6, 129), age and body mass (Table 1). Studies were undertaken in accordance with guidelines on the operation of the Animals (Scientific Procedures) Act 1986.

2.2. Drugs and chemicals

Constituents for the Krebs Henseleit buffer (NaCl 118 mM, Na₂CO₃ 24 mM, d-Glucose 10 mM, KCl 4 mM, NaH₂PO₄ 1.0, Na₂EDTA 0.5 mM, MgCl₂ 1.2 mM, CaCl₂ 2.5 mM) were purchased from BDH Laboratory supplies. S-Nitroso N-acetyl penicillamine (SNAP) and triphenyl tetrazolium chloride (TTC) were purchased from Sigma (Dorset, UK).

2.3. Langendorff perfusion

The method of Langendorff perfusion of mouse heart used in this study has been previously described [27,28]. In brief, mice were anaesthetised and anti-coagulated by the co-administration of 60 mg/kg sodium pentobarbitone and 100 IU heparin i.p. With the demonstration of adequate depth of anaesthesia, the heart was excised and immersed in ice cold buffer. The aorta was then cannulated (21 gauge), the heart transferred to the Langendorff apparatus and retrograde perfused at constant pressure (110 mm Hg) with Krebs Henseleit buffer, oxygenated with 95% O₂/5% CO₂ gas mixture (pH 7.4). A temperature probe inserted into the right ventricle enabled maintenance of normothermia (37°C) throughout the experimental protocol. Hearts were paced at the electrical threshold at 600 beats per minute throughout stabilisation and for the latter 20 min of the 30 min reperfusion period with electrodes in the left atria and on the aortic cannula. In the event of an arrhythmia, the rhythm was recaptured by a transient increase in the pacing threshold. No hearts were excluded secondary to intractable arrhythmia. End systolic and diastolic tensions were recorded with the use of a linear force transducer (Scame model GM3) attached to the heart’s apex with a 3/0 silk tie.
Table 1
Morphometrics and baseline functional parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Heart weight (mg)</th>
<th>Baseline coronary flow (ml/min)</th>
<th>Baseline contractile function (g/beat/min)</th>
<th>Baseline resting tension (g)</th>
</tr>
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<tbody>
<tr>
<td>Wild type control</td>
<td>26.99 (±1.21)</td>
<td>170 (±16)</td>
<td>3.32 (±0.13)</td>
<td>1465 (±187.3)</td>
<td>1.04 (±0.06)</td>
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<td>(n = 6)</td>
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<tr>
<td>Knockout control</td>
<td>23.86 (±0.99)</td>
<td>163 (±11)</td>
<td>3.00 (±0.20)</td>
<td>1484 (±168.9)</td>
<td>1.14 (±0.04)</td>
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<td>(n = 7)</td>
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<tr>
<td>Wild type 4PC</td>
<td>26.97 (±1.79)</td>
<td>172 (±9)</td>
<td>3.28 (±0.14)</td>
<td>1697 (±199.0)</td>
<td>1.04 (±0.05)</td>
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<td>(n = 6)</td>
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<tr>
<td>Knockout 4PC</td>
<td>24.96 (±0.38)</td>
<td>158 (±9)</td>
<td>2.78 (±0.16)</td>
<td>1217 (±142.5)</td>
<td>1.06 (±0.04)</td>
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<td>(n = 6)</td>
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<tr>
<td>Wild type SNAP</td>
<td>27.79 (±1.36)</td>
<td>180 (±9)</td>
<td>3.33 (±0.08)</td>
<td>1553 (±149.2)</td>
<td>1.04 (±0.05)</td>
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<td>(n = 6)</td>
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<tr>
<td>Knockout SNAP</td>
<td>27.30 (±0.81)</td>
<td>182 (±3)</td>
<td>3.03 (±0.23)</td>
<td>1364 (±240.4)</td>
<td>1.04 (±0.05)</td>
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<td>(n = 6)</td>
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<tr>
<td>Wild type 3PC</td>
<td>23.56 (±1.27)</td>
<td>166 (±13)</td>
<td>2.48 (±0.20)</td>
<td>1420 (±192.7)</td>
<td>1.15 (±0.13)</td>
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<tr>
<td>Knockout 3PC</td>
<td>24.52 (±0.69)</td>
<td>171 (±7)</td>
<td>2.59 (±0.10)</td>
<td>1100 (±88.6)</td>
<td>1.09 (±0.04)</td>
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<td>(n = 6)</td>
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<tr>
<td>Wild type 2PC</td>
<td>26.28 (±0.81)</td>
<td>153 (±6)</td>
<td>2.98 (±0.18)</td>
<td>1175 (±156.9)</td>
<td>1.17 (±0.08)</td>
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<td>(n = 6)</td>
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<tr>
<td>Knockout 2PC</td>
<td>25.27 (±0.92)</td>
<td>157 (±7)</td>
<td>2.47 (±0.17)</td>
<td>1250 (±176.1)</td>
<td>1.10 (±0.10)</td>
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<td>(n = 6)</td>
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No significant differences were noted between groups in any of the morphometric or baseline function data.

2.4. Experimental protocols

The study was divided into three groups, summarised in Fig. 1. In groups A and C, WT and KO hearts were subjected to ischaemic preconditioning regimes (PC) consisting of between two and four cycles of 5 min global ischaemia followed by 5 min reperfusion. Control hearts received time-matched perfusion prior to the index ischaemia. All groups were subjected to 35 min of global, normothermic no-flow index ischaemia and reperfused for 30 min, prior to infarct size determination as described below. Thirty minutes reperfusion has previously been demonstrated to be adequate for accurate TTC assessment of infarction in this model [27,28]. In the SNAP treatment group B, SNAP was dissolved in the Krebs Henseleit perfusion buffer (2 μM), and was therefore present throughout the ischaemia/reperfusion protocol.

2.5. Determination of infarct size

Infarct size was determined by TTC staining. The hearts were incubated for 10 min at 37°C with 1% TTC in phosphate buffer (pH 7.4), weighed, frozen, cut into transverse slices and fixed in 10% formaldehyde for 24 h. Infarcted and viable tissue appear pale and brick red, respectively. The individual slices were digitally photographed and the images imported into a graphics package (NIH Image v1.61) for planimetry. Infarct size for each heart is expressed as a percentage of risk volume (total ventricular volume, minus cavity spaces).

2.6. Statistical analysis

All data are expressed as mean±standard error of the mean (S.E.M.). Statistical analysis was performed using commercially available statistics software (StatView 4.5.1, Abacus Concepts). Data between groups was compared using factorial analysis of variance (ANOVA). Where significant, Fisher’s protected least significance difference (PLSD) post hoc test was applied. For comparison of data sets recorded over a period of time, ANOVA for repeated measures was used, and where significance was determined, the Fishers PLSD post hoc test was applied. Association between data was tested by Spearman rank correlation and linear regression was used to analyse the association for each group. Differences between groups were considered significant if the measured P-value was less than 0.05.

3. Results

3.1. Is eNOS activity pivotal for early ischaemic preconditioning?

To determine whether eNOS is essential for early ischaemic preconditioning, hearts from eNOS wild type and knockout animals were subjected to a robust four cycle, 5 min ischaemia and 5 min reperfusion preconditioning regime (protocol, Fig. 1A). The hearts were then subjected to 35 min of normothermic global index ischaemia followed by 30 min reperfusion, as described,
prior to determination of infarct size by triphenyl tetrazolium staining. If eNOS is pivotal to early ischaemic preconditioning, then even with a robust preconditioning regime, no protection would be expected in the eNOS knockout group. However, significant protection was observed in both wild type and knockout groups, with infarct reduction from 35±2 to 21±3% and from 31±2 to 23±3% in the wild type and knockout groups, respectively (data summarised in Fig. 2). Moreover, this protective effect was reflected in a significant reduction of contractile dysfunction, as summarised in Fig. 3. Therefore, eNOS is considered not to be essential to trigger/mediate the cardioprotection derived from early ischaemic preconditioning. This observation does not exclude a role for nitric oxide derived from eNOS to contribute to the preconditioning threshold.

3.2. Can exogenous nitric oxide protect eNOS wild type and knockout hearts from lethal ischaemia and reperfusion?

To confirm that the mechanisms for nitric oxide mediated resistance to ischaemia reperfusion injury exist in both wild type and knockout hearts, naive hearts were subjected to index ischaemia and reperfusion in the presence of SNAP (2 μM) (protocol, Fig. 1B). In both SNAP treated groups, significant attenuation of infarction was observed (summarised in Fig. 4), with wild type infarct size reduced to 15±1% (P < 0.001 versus control) and knockout infarct size to 17±3% (P < 0.001 versus control). Therefore, whilst early ischaemic preconditioning may not be dependent upon the synthesis of nitric oxide...
Fig. 4. Exogenous nitric oxide and infarct size limitation. 2 μM SNAP resulted in significant limitation of infarct size in both wild type and knockout groups compared to their respective controls.

from eNOS, exogenous nitric oxide can nonetheless imbue significant resistance to ischaemia/reperfusion injury in both wild type and knockout animals.

3.3. Does eNOS contribute to the threshold for triggering preconditioning?

To determine whether eNOS contributes to the threshold to preconditioning, the preconditioning stimulus was progressively reduced from four to three to two cycles (4PC, 3PC and 2PC, respectively) of 5 min ischaemia and 5 min reperfusion (protocol Fig. 1C). By reducing the number of preconditioning cycles, the hypothesis that eNOS contributes to the preconditioning threshold can be tested. If the protection is lost in eNOS knockout hearts, and yet preserved in the eNOS wild type hearts with the less robust preconditioning regimes then eNOS can therefore be said to be contributing to the preconditioning threshold.

In wild type hearts, ischaemic preconditioning with two, three and four cycles (i.e. in all preconditioning regimes) resulted in significant attenuation of infarct size (control 34±2%, 2PC 27±2%, 3PC 26±3%, 4PC 21±3%, for 2PC, 3PC and 4PC, respectively Fig. 5A). However, ischaemic preconditioning with fewer than four cycles of transient ischaemia/reperfusion failed to result in significant attenuation of infarct size in hearts from knockout mice. Whilst four cycles resulted in significant attenuation of infarct size (as shown in Fig. 2), the same did not hold true for two or three cycles (control 30±9%, 2PC 29±2%, 3PC 33±2%, 4PC 23±3%, respectively, for 2PC, 3PC and 4PC, Fig. 5B).

These data suggest that there is a role for eNOS contributing to the triggering of the preconditioning response, lowering the ischaemic threshold to preconditioning in wild type hearts compared to knockout hearts.

Fig. 5. Two and three cycles of preconditioning fail to protect eNOS knockout hearts. Reducing the number of cycles of preconditioning from four cycles (4PC) to three cycles (3PC) to two cycles (2PC) had no effect upon the protection observed in hearts from eNOS wild type mice (A). However, the protection in hearts from eNOS knockout mice (B) was lost with the less robust preconditioning regimen of less than four cycles (4PC).

4. Discussion

A number of recently published studies provide evidence that endogenous nitric oxide synthesis is not contributory to ischaemic preconditioning, which is in accordance with earlier pharmacological studies from both our own group [8], and others [9,10]. The evidence presented in this paper initially appears inconsistent with this data. However, we believe that instead of contradicting earlier reports, this data elaborates upon these earlier findings, and bridges to investigations implicating a role for nitric oxide...
in early over-drive pacing preconditioning [11] and preconditioning in other organ systems, such as kidney [29] and brain [30].

Exogenous nitric oxide has previously been shown to trigger preconditioning [6], and here we demonstrate that even in the absence of eNOS, exogenous nitric oxide, derived from the spontaneous nitric oxide donor SNAP, results in significant attenuation of infarct size. This result confirms that downstream targets of nitric oxide remain intact in the absence of eNOS, targets that may be pivotal in mediating the protection following less stringent preconditioning stimuli in wild type hearts.

Four cycles of preconditioning is equally efficacious in effecting significant infarct size limitation in both eNOS wild type and knockout hearts. Interestingly, a less robust preconditioning regimen (three or two cycles) results in no protection in eNOS knockout hearts and yet the wild type hearts demonstrate preconditioning triggered protection with these regimens. The data therefore suggest that enzymatic products of eNOS activity may contribute towards the multi-factorial trigger signal required to trigger/mediate early ischaemic preconditioning. This observation may have two explanations. The first may involve the magnitude of the preconditioning stimulus in this investigation compared to other early ischaemic preconditioning studies in other species [6–10]. At supra-threshold levels, ischaemic preconditioning may not obligatorily require the eNOS derived nitric oxide component of the preconditioning signal to trigger protection. However, the contribution provided by nitric oxide may become critical when the magnitude of the preconditioning stimulus is close to the preconditioning threshold. A precedent exists for this hypothesis: the involvement of free radicals in early ischaemic preconditioning. Baines et al. demonstrated that free radicals were essential for preconditioning elicited by one cycle but not four cycles of 5 min ischaemia/5 min reperfusion in the rabbit [31]. The second possibility concerns the catalytic properties of eNOS itself. Nitric oxide is not the sole product of eNOS; under conditions of low intracellular BH_{4} or L-arginine depletion, superoxide radicals may be generated [32,33]. Whilst L-arginine depletion is unlikely to occur during the relatively short duration of these experiments, neither BH_{4} nor free radicals were measured in this investigation. Therefore, the possibility of free radicals synthesised as a by-product of eNOS activity contributing to the preconditioning ‘signal’ cannot be excluded in the current study. The contribution of nitric oxide relative to free radical synthesis in the paradigm of preconditioning warrants further investigation.

In this study, we have chosen to investigate the role of nitric oxide in early ischaemic preconditioning using transgenic animals displaying a targeted knockout of the eNOS gene. This model has advantages over previous pharmacologically based studies insofar as it enables us to delineate the role of a single isoform of NOS that cannot be manipulated specifically pharmacologically. We cannot however exclude the possibility that there may be compensatory up-regulation of other NOS isoforms (as suggested in work by Kanno et al. [34]) to contribute to the preconditioning stimulus to bring about protection in the four cycle preconditioning protocol. However, given that there is at least a two-fold increase in the preconditioning threshold in the eNOS knockout animals, the hypothesis that there is a potential protective role of eNOS derived NO in triggering/mediating early preconditioning appears to hold true.

The observation that eNOS may contribute to early preconditioning may have potential importance in the clinical application of preconditioning in man. In patient based studies, patients at risk of, or who have recently suffered from, acute coronary infarction appear significantly more likely to have eNOS polymorphisms (G→T polymorphism in exon 7 of the gene which encodes a Glu→Asp amino acid substitution at residue 298 of eNOS) [35]. Such a polymorphism may adversely affect the nitric oxide generation resulting from a transient ischaemic insult, and may therefore attenuate protection potentially afforded by preconditioning. There are also implications with respect to gender. 17β Oestradiols are associated with eNOS expression and activity. Oestradiol administration results in both an increase of nitric oxide mediated vasodilatory response observed in the coronary vasculature of isolated perfused guinea pig heart [36] and preserves endothelial function and reduces frequency of arrhythmias after 15 min regional ischaemia in male dogs [37]. Furthermore, oestrogen receptor-α knockout mice had reduced myocardial viability associated with reduced reperfusion coronary flow compared to their wild types following a 45 min global ischaemic insult, associated with diminished NO metabolite accumulation [38]. However, data from rat and dog suggest that gender does not influence infarct size [39,40]. Nonetheless, eNOS may prove to be a useful pharmacological target in cardioprotection from acute coronary syndromes, and the eNOS genotype may prove to be a useful morbidity/mortality predictor in cases of acute myocardial infarction.

Acknowledgements

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