1. Introduction

In 1986 Furchgott first suggested that endothelial-derived relaxing factor might be nitric oxide (NO) [1]. Since then the remarkable role of nitric oxide (NO) as a modulator of biological phenomena has led to the question of its involvement within the spectrum of cardiovascular disease. The resulting research has implicated NO in atherosclerosis, hypertension, cardiomyopathy, pre-eclampsia, endotoxaemia [2] and cardiac allograft rejection [3] providing important additional insights into the pathogenesis of vascular and heart muscle disease. Although initially characterised in the vasculature NO is present in heart muscle [4] whilst the relative expression of the three isoforms (ncNOS, iNOS, ecNOS) responsible for its synthesis may have important implications in disease pathology. Apart from a cytotoxic role, NO has a role in cytostasis both regulating normal homeostasis and protecting against cell injury [5]. This, has led to the hypothesis that NO is cardioprotective in ischaemic heart disease. In testing this hypothesis very recent research has implicated NO in the phenomenon of ischaemic preconditioning. The current evidence suggesting its involvement in this exciting field is the focus of this review.

2. Background

Ischaemic preconditioning (PC) describes the profound myocardial protection that follows a short period of sublethal ischaemia and was recently considered by a NHBLI workshop to be the most powerful intervention to reduce myocardial infarct size other than reperfusion [6]. Initially described by Murray et al. in 1986 [7], in trying to understand the metabolic consequences of ischaemia, they found that brief periods of repetitive ischaemia in a canine model produced significant protection against myocardial cell death. There appears to be a bimodal distribution of protection with an initial more powerful but short-lived phase occurring between 1 and 3 h (‘Early Preconditioning’) and a delayed less powerful phase occurring between 12 and 72 hours (‘Late Preconditioning’) after the initial ischaemic insult [8]. A number of important ligands have been implicated as both triggers and mediators including adenosine [9], acetylcholine [10] and bradykinin [11]. Knowledge that these substances could also generate NO by activating the endothelial derived constitutive nitric oxide synthase enzyme (eNOS) have implicated NO as a potential trigger/mediator.

NO can act as an intermediary in the formation of peroxynitrite and hydroxyl radicals as well as other reactive oxygen species. Initially the anion oxonitrate is formed by the reduction of NO which quickly reacts with additional NO to form nitrate and nitrite. Further breakdown of nitrate and nitrite yields dinitrogen monoxide and hydroxyl ions. Oxonitrate may also combine with oxygen to produce peroxynitrite [12,13]. In addition NO can combine in a direct bimolecular reaction with superoxide ions in oxygenated media to form peroxynitrite [14]. Following the expression of the inducible NO synthase (iNOS) after myocardial ischaemia [15] these NO-derived radicals are thought to aggravate injury under certain circumstances. However, free radicals can also trigger PC since the administration of antioxidants during the brief triggering ischaemia has been found to prevent both early and late PC [16]. Thus a detrimental component of NO can paradoxically trigger subsequent protection. However, NO is also directly protective during myocardial ischaemia–reperfusion in other circumstances [17,18]. These promiscuous effects of NO within ischaemia–reperfusion are reinforced by a recent study [19] in the isolated heart where the NO donor sodium nitroprusside and the NO synthase inhibitor Ng-nitro-L-arginine (L-NOARG) both reduced ischaemia–reperfusion injury.
3. Early preconditioning

Vegh and colleagues in a canine model of pacing-induced arrhythmias showed that the anti-arrhythmic effect of PC was abolished by the NO pathway inhibitor N\textsubscript{G}-nitro-arginine methyl ester (L-NAME) [20] and methylene blue, a guanylate cyclase inhibitor [21]. Furthermore, the NO donors glyceryl trinitrate and 3-morpholino-sydnonimine-hydrochloride (SIN-1) have been shown to mimic the effects of PC in abolishing reperfusion-induced ventricular arrhythmias in the isolated rat heart [22]. These studies above suggest NO protects against ischaemia–reperfusion arrhythmia in the dog. However, using this phenomenon as a surrogate for PC is difficult as PC against ischaemia–reperfusion arrhythmia is not present in all species e.g. swine. Also the question of whether NO can protect against ischaemic cell death in early PC remains unclear.

Unpublished data from our laboratory in a simulated ischaemia model of early PC in rat neonatal cardiocytes suggests that the NO donor s-nitroso-N-acetyl-l,l-penicillamine (SNAP) given for 90 min prior to lethal hypoxia significantly protects against ischaemic injury. Also, the protective effect of PC is blocked by the NO synthase (NOS) inhibitor N\textsubscript{G}-monomethyl-l-arginine (LNMMA). If NO is important in early PC then endothelial NO generation in response to ischaemia must have an important role. Further experiments are required to further understand the endothelial–myocyte–NO interaction during the early phase of protection.

4. Late preconditioning

A delayed increase in NO production has been shown following ischaemia/reperfusion which coincides with the time course of late PC [23]. Subsequently recent research has implicated NO as both a trigger and mediator of late PC. Bolli and colleagues in conscious rabbits showed that late PC against stunning was inhibited by NOS inhibitors [24]. Of particular interest was the finding that the non-selective NOS inhibitor l-nitro-arginine (LNA) given at day one inhibited PC at day 2 whereas the selective inducible NOS inhibitors aminoguanidine and methylisothiourea given at day 2 inhibited PC at day 3. This suggests a role for both constitutive eNOS and iNOS in late PC, the expression and importance of which may be temporally regulated [25]. More recently the same group have reported that the infarct-sparing effect of late PC can also be blocked by LNA suggesting that NO synthase as a mediator of PC against myocardial infarction [26]. Parratt’s group have previously implicated iNOS in late PC where the protective effect of pacing against arrhythmias in dogs was abolished by dexamethasone, an inhibitor of iNOS [27]. This is supported by more recent data in a PC model assessing infarct size in anaesthetised rabbits after coronary occlusion. Both aminoguanidine and dexamethasone were found to completely block the infarct-limiting effect of PC [28].

5. Possible mechanisms of action

What has emerged from the PC field is that a variety of ligands and their corresponding receptors may act in an additive fashion to trigger protection. Our current understanding of the post-receptor cascade is embraced by the Downey hypothesis where by activated receptors couple to G proteins leading to the activation of protein kinase C (PKC) via the formation of diacylglycerol [29]. The complex downstream pathways are currently under investigation and are likely to involve modulation of various kinases including tyrosine and mitogen-activated kinases [30]. Several protein effectors have been described in the late phase of PC including heat shock proteins [31] but it is becoming increasingly clear that an end effector of PC is likely to be the ATP-sensitive potassium channel [32].

Circumstantial evidence exists implicating NO and its interactions in the signalling scheme of PC. However, unique properties of the NO–guanylate cyclase–cyclic guanosine 3,5-monophosphate (cGMP) pathway provide a parallel hypothesis whereby NO has an independent role in cardiac protection. The influence that NO may have in modulating the accepted signalling pathway underlying PC is represented in Fig. 1 with relevant evidence summarised below.

5.1. Free radical generation

Studies of ischaemia/reperfusion injury have highlighted the importance of NO as a free radical donor. Ischaemia/reperfusion generates calcium and NADPH both necessary for NO production together with superoxide anions [33]. NO combines with superoxide to generate peroxynitrite which then decomposes to yield the hydroxyl radical [34] and other reactive oxygen species. Interestingly, oxidative stress has been shown to directly activate extracellular signal related kinases in cardiac myocytes [35]. Also several studies have demonstrated activation of PKC by oxygen radicals [36] and hydrogen peroxide [37,38]. This provides a novel hypothesis by which NO derived reactive oxygen species may trigger the pathway leading to PC. Recent data lending further support for a role of NO-mediated free radical generation in late PC is provided by Bolli’s group who have found that the cardioprotective effects of NO are completely abrogated by the antioxidant mercaptopropionyl glycine, a scavenger of peroxynitrite and hydroxyl radicals [39].

5.2. Lipopolysaccharide

The observation that sublethal doses of bacterial endo-
toxin (lipopolysaccharide) can increase myocardial resistance to ischaemia and reperfusion was first made in 1989 by Brown et al [40] and subsequent research suggests this may be an important trigger for delayed protection, although the exact mechanism remains unclear [41]. Endotoxin is a powerful activator of iNOS [42] fueling recent interest in NO as the effector. Several preliminary reports in abstract form have implicated NO in PC experiments using monophosphoryl lipid A (MLA), a modified less pyrogenic lipopolysaccharide derivative. MLA causes protection by the induction of iNOS through tyrosine phosphorylation [43] and this effect can be abolished by L-NAME [44].

5.3. Adenosine

Adenosine, produced in response to ischaemia, can trigger PC in most species and is an important ligand in the Downey hypothesis. A relationship with NO must clearly exist as adenosine mono- and diphosphate are important activators of the calcium –dependent cNOS [45]. Indeed, in a model of PC against hepatic ischaemia–reperfusion injury adenosine has been shown to stimulate NO production to protect against hepatocellular injury [46]. Activation of iNOS has also been shown experimentally in cultured rat cardiac myocytes where interleukin-1-induced nitrite production is significantly increased by the addition of adenosine or chloroadenosine. This effect was not attenuated in the presence of a PKC inhibitor [47].

5.4. Protein kinase C

S-nitrosylation of protein thiols is one of the regulatory cellular mechanisms of nitric oxide [5]. Since PKC has critical thiol residues which determine its activity it is not surprising that NO can regulate this enzyme. Evidence for a negative regulatory effect of NO on PKC appears in other cell systems studied. For example in a B16 melanoma cell line NO gas, sodium nitroprusside and SNAP rapidly caused both reversible and irreversible inhibition of PKC predominantly by the inhibition of phorbol ester binding [48]. Conversely, PKC can modulate the actions of NO. Overexpression of rat PKC with a replication-incompetent retroviral vector in rat mesangial cells has been shown to increase iNOS expression assessed by immunoblotting [49]. However, a counter-regulatory role for PKC with cNOS is suggested in a study where the PKC inhibitors staurosporine or chelerythrine caused the increased expression of cNOS in bovine aortic endothelial cells [50]. Further studies are required to elucidate the exact relationship between NO and myocardial PKC isoforms thought to be involved in PC. The possible interactions between adenosine, PKC, NOS and NO are summarised in Fig. 2.

5.5. Heat shock protein upregulation

Heat shock proteins (HSP) are upregulated in response to ischaemic or heat stress and have been implicated as protein effectors in late PC [31]. Evidence is also emerging that NO can cause cytoprotection by inducing HSP expression. The NO donor SNAP has been shown to protect cultured rat hepatocytes from tumour necrosis factor-induced apoptosis by the induction of HSP70 [51]. Preliminary data involving an in vivo rabbit model of SWOP have shown that NO-induced protection is associated with an increase in HSP72 expression [52].
NO-dependent insulin release from pancreatic islet cells [60] and peripheral vascular vasodilatory responses [61,62] have been found to involve specific K-ATP channels. The finding that NO can potentiate the ATP sensitive K channel current in isolated guinea-pig ventricular cells [63] raises the distinct possibility that NO could modulate mitochondrial K-ATP channels in human myocardium and provides an exciting research challenge for the future.

5.8. Modulation of mitochondrial function

How opening of mitochondrial K-ATP channels confers protection is unknown but current hypotheses include (i) the reduction of calcium influx through the calcium uniporter and ATP conservation via (ii) promotion of binding of the endogenous ATPase inhibitor IF, [64] or perhaps via (iii) modulation of oxidative phosphorylation through the control of matrix volume. The latter may play an important role in the regulation of mitochondrial metabolism and this subject has been reviewed extensively [65], but in the context of NO this mechanism may have important implications. NO has been shown to reversibly inhibit cytochrome C oxidase but its oxidised product peroxynitrite irreversibly inhibits complexes I–III of the respiratory chain in brain submitochondrial particles [66]. Whether inhibition is reversible or irreversible depends on the availability of superoxide to form peroxynitrite, the presence of glutathione and this in turn determines cell viability.

Mitochondrial dysfunction is a critical component of ischaemia–reperfusion injury and is characterised by dissipation of the membrane potential, induction of the mitochondrial permeability transient and ATP depletion. A recent study has demonstrated mitochondrial adaptation to hypoxia through a partial and reversible inhibition of mitochondrial respiration which resulted in the maintenance of cell viability without net ATP depletion [67]. We have recently demonstrated reversible inhibition of respiration by NO in neonatal rat cardiocytes in culture without dissipation of the membrane potential (unpublished data). This phenomenon, through the reversible modulation of metabolism, may represent a physiological mechanism by which mitochondria metabolically adapt to hypoxia and may provide a plausible hypothesis to the cytoprotection conferred by NO.

6. Clinical perspective: the nitrate controversy

The principal beneficial effects of NO in reducing preload, augmenting collateral coronary flow and inhibiting platelet aggregation [42] provide a powerful theoretical basis for the use of nitrates in the routine treatment of ischaemic syndromes, myocardial infarction (MI) and heart failure. Despite this rationale the routine use of
nitrates as cardioprotective agents to reduce mortality remains uncertain.

Myocardial infarction: In a small number of MI trials predating the thrombolytic era intravenous nitrates were thought to have beneficial effects on infarct size and mortality [68–70]. Subsequent meta-analysis of these trials indicated up to a 49% reduction in mortality with the use of prolonged intravenous nitroglycerin [71,72]. However, the later and larger mega-trials such as ISIS-4 [73] and GISSI-3 [74] could not confirm a beneficial effect on mortality in contradiction to previously published data. One major criticism of the ISIS-4 and GISSI-3 mega-trials was the widespread use of open-label oral nitrates in the placebo and control groups (62% and 57% respectively) which may have diluted the true effects of nitrates in both studies [75]. Of course, the use of intravenous nitrates was not addressed in these studies and no study has specifically assessed the potential benefit of nitrate therapy before MI.

Angina: Despite clear demonstrable effects in relieving symptoms and electrocardiographic changes of ischaemia nitrates have not been shown to influence mortality in stable or unstable angina. The observation that pre-infarction angina may represent a correlate for PC in humans has been demonstrated in several studies but remains controversial. In one of the largest studies analysing patients in the Thrombolysis in Myocardial Infarction (TIMI) 4 study [76] the presence of pre-infarction angina reduced the combined end-points of in-hospital death, severe congestive heart failure or shock from 12% in patients with no preceding angina to 4%. As expected the use of oral anti-anginal medication was higher in the angina group prior to MI. In particular nitrate usage was 34% in the angina group compared to 14% in the no angina group and it is interesting to speculate as to the contribution of antecedent nitrate treatment to cardioprotection.

Heart failure: The beneficial haemodynamic effects of nitrates in preload reduction inducing a reduction in pulmonary and left ventricular end diastolic pressure are beneficial in heart failure. A recent study of the use of intravenous nitrates in acute severe pulmonary oedema showed a significant reduction in 7 day mortality [77]. No study has assessed the sole use of nitrates in chronic heart failure but the V-HeFT I and II trials [78] showed that the combination of isosorbide dinitrate and hydralazine produced a 38% reduction in mortality at 1 year compared to placebo or prazosin.

Cardiac surgery: Ischaemia–reperfusion injury is an important phenomenon during cardiac surgery and the pharmacological manipulation of cardioplegic solutions has emerged as a powerful clinical tool in the preservation of myocardial integrity [79]. Two studies have addressed the cardioprotective role of NO during cardiac surgery by blood cardioplegic supplementation with L-arginine [80] and SPM-5185, a cysteine containing NO donor [81]. These agents respectively produced both reduced infarct size and improved post-ischaemic contractile performance.

If NO can precondition the myocardium then nitrates may also have a protective role before routine cardiac surgery.

7. Future strategies

From our understanding of experimental and clinical preconditioning and the emerging evidence for the role of NO in cardioprotection perhaps the emphasis should now be shifted to extensively re-exploring a role for nitrates before myocardial necrosis has occurred. Large properly designed trials in the context of stable and unstable angina are required to answer this question. What is likely to be of paramount importance is the timing of therapy in relation to infarction, dose regimen and the concept of nitrate tolerance. Indeed, the largest pre-thrombolytic randomised trial of intravenous nitrates following MI by Jugdutt and Warnica showed that the infarct size-reducing effect of nitroglycerin was most marked when therapy was begun less than 4 hours from the onset of pain and critically dependent on dosage [82]. Future therapeutic strategies are likely to involve the development of more stable NO donors, the adjunctive use of free radical scavengers and the pharmacological use of agents downstream of NO. Thus, a cardioprotective role for the selective type-5 cGMP phosphodiesterase inhibitor sildenafil (Viagra) may yet to be found.

NO is emerging as an important cytoprotective agent and may play a pivotal role in myocardial protection both as a trigger and mediator of PC. The role of nitrates in the clinical setting needs to be re-addressed in the context of a therapeutic strategy prior to MI before excluding the possibility of real benefits in terms of infarct size and mortality. The recent description of a physiologically relevant murine model of early and late ischaemic PC [83] will provide a powerful tool for the future use of genetic engineering in elucidating the cellular mechanisms of preconditioning and the role of NO. Thus a greater scientific understanding of the basis of NO-induced cytoprotection against ischaemic injury may help to answer these important clinical questions.

References


[34] Beckman JS, Beckman TW, Chen J, Marshall PA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–1624.


[67] Lizardoa M, Moro MA, Knowlles RG et al. Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. Biochim Biophys Acta 1996;134:877–880.


