Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes

ROBY D. RAKHIT,1 RICHARD J. EDWARDS,1 JAMES W. MOCKRIDGE,1 ANWAR R. BAYDOUN,1 AMANDA W. WYATT,2 G. E. MANN,2 AND MICHAEL S. MARBER1

Department of 1Cardiology, St. Thomas’ Hospital, and 2Department of Physiology, Centre for Cardiovascular Biology and Medicine, Kings College, London SE1 7EH, United Kingdom

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excess of 80% of cells were beat spontaneously for the duration of the experiment. Experiments were performed after 2 days in culture.

Simulated Ischemia Model

Cells were washed with PBS before addition of 1 ml ischemia buffer (in mM: 118 NaCl, 24 NaHCO₃, 1 NaH₂PO₄·H₂O, 2.5 CaCl₂·2H₂O, 1.2 MgCl₂, 0.5 sodium EDTA·2H₂O, 20 sodium lactate, and 16 KCl, pH 6.2). Near-anoxic conditions were achieved by one of two methods. In the PC experiments, after pregassing with 95% argon-5% CO₂, the ischemia buffer was added to the cells, which were then placed in a purpose-built ischemia chamber and incubated at 37°C in 95% argon-5% CO₂. The O₂ content of the atmosphere inside the chamber was <1% for the duration of the experiment, as measured by an on-line meter (Griffin and George, Fife, UK). Subsequent experiments were performed using the BBL GasPak Pouch System (Becton-Dickinson). This system provides a compact microenvironment contained in an impermeable bag that, when properly activated and sealed, provides anaerobic conditions with an O₂ concentration of <2% within 2 h of incubation at 35°C. First, the liquid-activating reagent (containing 5 g of the following: iron powder, calcium carbonate, citric acid, and inert extender) is placed in the reagent channel of the GasPak Pouch. Next, the 6- or 12-well plate is heat-sealed within the bag. The catalytic reaction that ensues consumes O₂ and produces CO₂. An anaerobic strip on the surface of the bag turns from blue to white once anoxic conditions are achieved. Both methods for producing hypoxia after 6 h incubation at 37°C in 95% air-5% CO₂ produced ~50% cell death in control cells.

Evaluation of Cell Viability

Cell viability was quantified using 3–4,5-di-methylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) bioreduction assay (23). Briefly, cell culture plates were washed with PBS, exposed to 5 mg/ml of MTT solution, and placed in an incubator at 37°C for 20–30 min. The dark blue crystals that formed were dissolved in stop solution containing 0.1 M HCl, 10% Triton X-100, and isopropanol, and the absorbance was read at 570-nm wavelength.

Measurement of Lactate Dehydrogenase Activity

After the end of simulated ischemia, buffer was gently aspirated and saved for lactate dehydrogenase (LDH) determination. A spectrophotometric LDH enzyme assay was performed with a Sigma assay kit (TOX-7; see Ref. 16).

Experimental Protocols

In all experiments, cell viability was assessed by MTT bioreduction and LDH release at the end of lethal ischemia (Fig. 1).

Experiment 1: PC. Lethal ischemia was simulated by subjecting the cells to 6 h of simulated ischemia (SI) using either the hypoxia chamber or GasPak Pouches. Cells were “preconditioned” with 90 min of ischemia followed by 30-min “reperfusion” in normal maintenance medium before 6 h of SI.

Experiment 2: Effects of L-NMMA and dexamethasone on PC. The effects of L-NMMA (1 mM) and dexamethasone (0.1 µM) on PC were studied. At this concentration, dexamethasone has previously been shown to completely inhibit iNOS mRNA expression in rat neonatal cardiocytes (35). L-NMMA was given during PC ischemia, and dexamethasone pretreatment was given 1 h before PC ischemia.

Experiment 3: SNAP-induced protection. Cells were exposed to 90 min of SNAP (100 µM-2 mM) dissolved in control buffer (in mM: 118 NaCl, 24 NaHCO₃, 1 NaH₂PO₄·H₂O, 2.5 CaCl₂·2H₂O, 1.2 MgCl₂, 0.5 sodium EDTA·2H₂O, 20 sodium pyruvate, 2 D-glucose, and 10 and 4 KCl, pH 7.4) followed by 30 min of “reperfusion” to mimic PC. The effects of 90 min SNAP exposure alone (100 µM-2 mM) were also assessed to exclude toxicity.

Fig. 1. Experimental protocols. The following experiments were performed on neonatal rat ventricular myocytes in primary culture. Experiment (Expt) 1 represents preconditioning (PC) cells “preconditioned” with 90 min of simulated ischemia (SI) before 6 h SI. Expt 2 shows effects of 1 mM N⁵-monomethyl-l-arginine monoacetate (L-NMMA) and 0.1 µM dexamethasone on PC. Expt 3 is S-nitroso-N-acetyl-l-arginine-L-penicillamine (SNAP)-induced protection cells pretreated 90 min with 1 mM SNAP to mimic PC before 6 h SI. Expt 4 shows effects of 10 µM 1H[1,2,4]oxadiazolo[4,3-d]quinoxalin-1-one (ODQ), 2 µM chelerythrine, 10 µM glibenclamide, and 100 µM 5-hydroxydecanoate (5-HD) on SNAP-induced protection. Cell viability was assessed by MTT activation and LDH release at end of 6 h SI, as described in METHODS. Isch, ischemia; Dex, dexamethasone; R, reperfusion; MTT, 34,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.
Experiment 4: Effects of ODQ, chelerythrine, glibenclamide, and 5-HD on SNAP-induced protection. The effects of the pharmacological inhibitors ODQ (10 µM), chelerythrine (2 µM), glibenclamide (10 µM), and 5-HD (100 µM) on SNAP (1 mM)-induced protection were assessed. ODQ and chelerythrine were given during SNAP exposure and before long ischemia. The high dose of glibenclamide was chosen because previously it has been shown to block sarcolemmal ATP-dependent K⁺ channel activity in rat ventricular cells. However, lower doses have been shown to block mitochondrial ATP-dependent K⁺ channels (9). 5-HD at the dose used has been shown to inhibit mitochondrial ATP-dependent K⁺ channel flux in isolated rat heart mitochondria (13). Glibenclamide and 5-HD were given throughout the experimental protocol as it is currently unknown at which stage sarcolemmal/mitochondrial ATP-dependent K⁺ channel opening is important for protection during PC.

Measurement of cGMP Levels

Confluent cardiac myocyte monolayers in 24-well plates were subjected to SNAP treatment (1 mM) for 90 min in the presence or absence of ODQ (10 µM). At the designated time, the experimental conditions were removed, and the cells were placed on ice and incubated with 0.1 M HCl (500 µl/well, 60 min). Cell extracts were stored at -20°C for RIA of cGMP levels, as previously described (31).

Western Blotting Analysis for iNOS Protein

Cells undergoing a PC protocol were harvested every 30 min from the beginning of PC ischemia at time points of 30, 60, and 90 min. Cells were washed three times with PBS, harvested in 1 ml of sample buffer (250 mM Tris·HCl, pH 6.8, 4% SDS, 10% glycerol, and 2% β-mercaptoethanol), and then boiled for an additional 5 min. The cell extracts were then centrifuged for 5 min to remove insoluble material. The samples were then loaded on a 7.5% polyacrylamide gel and after one-dimensional separation were transferred to nitrocellulose membranes (Hybond C, Amersham, UK). Uniform protein loading was confirmed by Coomassie staining of identically loaded gels. The blots were probed with a rabbit polyclonal antibody specific for iNOS and a peroxidase-conjugated swine anti-rabbit IgG secondary antibody before detection with enhanced chemiluminescence (ECL, Little Chalfont, UK). Mouse macrophage cell lysate was used as a positive control.

Statistical Analysis

Data are expressed as means ± SE. The n numbers refer to the number of wells from which data were obtained from at least four separate experimental preparations. For the cell viability/LDH data, comparisons between groups were analyzed using a parametric one-way ANOVA combined with the Tukey-Kramer multiple comparisons test. cGMP data were analyzed using the Kruskal-Wallis nonparametric one-way ANOVA combined with Dunn's multiple comparisons test (Graphpad Instat 1998). A P value of <0.05 was considered to be statistically significant.

RESULTS

PC of Rat Neonatal Cardiomyocytes: Effects of L-NMMA and Dexamethasone

PC with 90 min of ischemia protected cardiomyocytes against subsequent 6 h of lethal ischemia, resulting in a significant 28.8% increase in MTT activation and 14.5% reduction in LDH release compared with controls shown in Fig. 2. This protection was blocked by the nonspecific NOS inhibitor L-NMMA (1 mM), causing a significant reduction in MTT activation/increase in LDH release compared with preconditioned cells. PC in the presence of L-NMMA was associated with an unexpected further increase in LDH release compared with controls, suggesting a worsening of ischemic injury. Dexamethasone (0.1 µM) pretreatment did not have any effect on PC-induced protection. Neither L-NMMA nor dexamethasone alone had any effect on cell viability either when given alone or after 6 h of ischemia.

Effect of 90-min SNAP Treatment to "Mimic" PC: Effects of ODQ, Chelerythrine, Glibenclamide, and 5-HD

The effect of treatment for 90 min with the NO donor SNAP to mimic PC is shown in Fig. 3A. A 90-min exposure alone with SNAP (100 µM and 1 mM) produced no deleterious effect on cell viability compared with untreated controls. However, SNAP (2 mM) was associated with cytotoxicity. When cells were subjected...
to SI after exposure (Fig. 3B), both 100 µM and 1 mM SNAP displayed protective effects, with 1 mM SNAP producing a profound and significant protection against ischemic cell death, increasing MTT bioreduction by 64% and reducing LDH release 31.1% compared with controls (see Fig. 4). This protection did not differ significantly from that afforded by PC. SNAP-induced protection was completely abolished by ODQ (10 µM) but was unaffected by the presence of chelerythrine (2 µM), glibenclamide (10 µM), or 5-HD (100 µM; Fig. 4). None of the inhibitors given alone had any effect on cell viability after 6 h of ischemia.

cGMP During SNAP Treatment: Effect of ODQ

SNAP caused a significant rise in cGMP level compared with the control. This effect was abolished by ODQ (10 µM) and is shown in Fig. 5.

DISCUSSION

Interest in the involvement of NO in PC was initially fuelled by knowledge that (10) the ligands adenosine (24), ACh (40), and bradykinin (11), important triggers of PC, could also generate NO by activating the endothelial-derived NOS enzyme constitutive (c) NOS (22). Later, an understanding of the important role of free radicals in triggering PC (32) and the unique properties of NO both as a free radical and as a source of reactive oxygen species (29) further suggested NO as a potential target.

The biological effects of NO must be set in the context of physiological tissue concentrations to put any in vitro biological response into a truly physiologically relevant context. The NO donor SNAP was chosen since we felt it had the most stable pharmacokinetics in our cell culture system and had a sufficiently long half-life in the context of our experimental protocols. SNAP appears to be a low-output NO donor, and a 1 mM dose gives an effective concentration of 0.46 ± 0.2 µM after 20 min (6). This compares favorably with physiological concentrations of NO found in cardiovascular tissue (21). Therefore, we believe our SNAP responses to reflect physiologically relevant tissue levels of NO.

This study implicates NO as an important cardioprotective agent in a cellular model of early PC. Significant protection against simulated ischemia is seen both by PC and with the use of the NO donor SNAP when lethal simulated ischemia is applied only after 30 min of reperfusion. This observation correlates with the time course of early PC, and to our knowledge this is the only study so far documented implicating NO directly in early PC against ischemic cardiomyocyte death. Parratt (25) has previously hypothesized that endothelially derived NO from the hypoxia-induced activation of cNOS could act as a trigger for PC of the myocardium. What is striking is that NO is able to mediate protection in isolated myocytes in the absence of juxtaposed endothelial cells, suggesting an independent myocyte-derived pathway for NO generation during PC. Our data suggest that myocyte NOS mediates PC, as the protection afforded by PC is blocked by the nonspecific NOS inhibitor L-NMMA given during PC ischemia. Furthermore, the fact that protection is unaffected by dexamethasone pretreatment (which inhibits iNOS mRNA transcription) and that iNOS protein expression is not detected during PC ischemia suggests that protection is mediated by a Ca²⁺-dependent/cNOS isoform. SNAP mimics PC, thus implicating NO as a trigger. We have further studied the downstream mechanisms of SNAP-triggered PC by studying the effects of the specific guanylyl cyclase inhibitor ODQ, the specific PKC inhibitor chelerythrine, and the inhibitors of sarcolemmal/mitochondrial ATP-sensitive K⁺ channels, glibenclamide and 5-HD, respectively. The mechanism of SNAP-induced protection appears to be cGMP
dependent but independent of PKC or sarcolemmal/mitochondrial ATP-sensitive K⁺ channel opening. In addition, by demonstrating that exogenous NO results in elevated cGMP levels using RIA, we have further implicated cGMP in the mechanism of NO-induced early PC.

Few studies have implicated a role for NO in early PC, and these largely involve animal models of protection against pacing (36, 37) and reperfusion-induced arrhythmias (1). In studies involving the effect of PC on reperfusion-induced arrhythmias in the isolated rat heart, Bilinska et al. (1) showed that NO donors were able to mimic PC. However, Lu et al. (20) failed to implicate NO, as the inhibitors L-NMMA and nitro-L-arginine methyl ester did not have any effect on PC-induced protection. In this latter study, the NOS inhibitors were given before and not during PC ischemia, as in our study, which may explain the negative result. The majority of work in the field has focused on the role of NO in late or second-window PC, and this has now been almost completely characterized. This work has been recently reviewed by Bolli et al. (3). The NO hypothesis states that “late PC involves the sequential activation of different NOS isoforms in a time dependent fashion with endothelial NOS generating the NO

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![Fig. 4. Effects ODQ, chelerythrine, glibenclamide, 5-HD on SNAP-induced PC. Protective effect of pretreatment with 1 mM SNAP was studied in the presence or absence of ODQ (10 µM), chelerythrine (2 µM), glibenclamide (10 µM), and 5-HD (100 µM). Inhibitors were given as described in Experimental Protocols (Fig. 1). ***P < 0.001.](image)

![Fig. 5. cGMP levels measured by RIA expressed as percentage of control untreated cells (100%) in cells exposed to 90 min of treatment with 1 mM SNAP. Effect of 10 µM ODQ is also shown. **P < 0.01 *P < 0.05.](image)

![Fig. 6. Western blot probed with anti-inducible nitric oxide synthase (iNOS) polyclonal rabbit antibody. Cells were harvested for protein at 30, 60, and 90 min during PC ischemia. Mouse macrophage cell lysate was used as positive control.](image)
that initiates the development of PC on day 1 and iNOS then generating the NO that protects against recurrent ischemia on day 2. “The mechanism is oxidant sensitive and PKC dependent. Our results do not implicate a role for iNOS and differ importantly from these findings. In the light of our data, we would propose that the mechanism of early PC by NO is cNOS dependent and is mediated via cGMP. A clear rationale exists for a protective role for cGMP against ischemia-reperfusion injury. cGMP may act by 1) reducing the influx of cellular Ca2+ through L-type Ca2+ channels or 2) stimulating a cGMP-sensitive phosphodiesterase with a resultant reduction in levels of cAMP (25). This together with the known effect of NO in reducing myocyte contractility (5) would serve to reduce oxygen consumption and energy demand. A role for cGMP is further supported by a recent study that has demonstrated increased cGMP levels in the isolated perfused rat heart (18). However, the exact mechanism of NO-induced cytoprotection remains a subject of great debate. NO is not only an important pathological mediator but also a powerful physiological regulator, and a better understanding of these dichotomous effects is required. Recent research suggests that redox state (17), antioxidant effects via the action of hemoxygenase (30), the regulation of apoptosis (14), and the regulation of myocardial oxygen consumption through modulation of mitochondrial function (19, 39) may underlie the basis of NO-induced cytoprotection. In conclusion, this study shows that NO is a powerful trigger for early PC in isolated myocytes and that the PC effect is mediated probably by a cNOS isoform but is independent of iNOS. The mechanism of protection is dependent on cGMP, and we have demonstrated an NO-induced increase in cGMP synthesis that may lead to a number of physiological effects resulting in cytoprotection. This study further adds to evolving experimental research indicating a significant cardioprotective role for NO against ischemia-reperfusion injury and should herald the reassessment and development of new pharmacological strategies for drugs with NO-modulating properties in the clinical management of ischemic heart disease.

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