Role of the L-arginine/nitric oxide pathway in ischaemic/reoxygenation injury of the human myocardium

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ABSTRACT

The role of the L-arginine/nitric oxide (NO) pathway in myocardial ischaemic/reperfusion injury remains controversial in experimental animal models. The aim of the present studies was to investigate the role of this pathway in the human myocardium. Myocardial specimens from right atrial appendages of patients undergoing elective coronary bypass graft surgery were incubated in crystalloid buffer at 37°C and subjected to 120 min of simulated ischaemia followed by 120 min of reoxygenation. Tested drugs were added 15 min before ischaemia, and maintained during ischaemia and throughout reoxygenation. Ischaemia resulted in severe myocardial damage, as assessed by the leakage of lactate dehydrogenase (LDH) into the incubation medium and by the capacity of the tissue to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan product. L-Arginine (10 mM), a precursor of NO, significantly decreased LDH leakage (from 9.0±0.6 to 5.3±0.3 units/g wet wt.; P<0.05), but had no effect on MTT reduction or oxygen consumption. D-Arginine (10 mM), N⁵-nitro-L-arginine methyl ester (L-NAME; 0.5 mM), an NO synthase inhibitor, and S-nitroso-N-acetylpenicillamine (at 1, 100, 500 and 1000 μM), an NO donor, had no significant effects on the measured indices, and L-NAME did not reverse the protection afforded by L-arginine against LDH leakage. In addition, the formation of nitrotyrosine was not influenced by ischaemia/reoxygenation alone or by the agents investigated. In conclusion, these data suggest that L-arginine affords modest protection against ischaemic/reoxygenation injury of the human myocardium, an action that is NO-independent, and that NO metabolism does not play a significant role in this model.

INTRODUCTION

The pathophysiology of cardiac injury induced by ischaemia and reperfusion is probably multifactorial and is not yet well understood, but changes in nitric oxide (NO) metabolism [1–18], as well as the production of oxygen-derived free radicals [19–21], are thought to play a critical role. NO is a ubiquitous molecule that possesses a number of physiological actions, including a potent vasodilatory effect [22], anti-platelet activity [23,24] and inhibition of neutrophil aggregation and adhesion [25]. NO is also known to be involved in pathological processes other than ischaemia/reperfusion injury, such as septic shock [26], atherosclerosis [27], inflammatory...
myocarditis [28], heart failure [29] and acute allograft rejection [30]. The role of NO and its metabolites in myocardial ischaemic/reperfusion injury has been investigated in a number of studies, with variable results. Whereas some have found that NO precursors and NO-releasing agents are cardioprotective [1–7], others have shown that they may exacerbate injury, an action that is reversed by inhibitors of NO synthase (NOS) [8–13]. Therefore the role of NO in ischaemic/reperfusion injury remains controversial. Moreover, to the best of our knowledge, the effect of NO on the ischaemic and reperfused human myocardium has not been reported.

This lack of information on the action of NO in humans contrasts with the fact that most patients presenting with cardiac ischaemic syndromes and patients undergoing cardiac surgery receive NO donors. The mechanisms by which NO exerts beneficial or detrimental effects in the various experimental settings are also unclear. It has been shown that NO inhibits cardiac myocyte contractility [31], an effect that may be attributed to its ability to modulate mitochondrial respiration in different ways: (i) by inactivation of iron–sulphur–containing complexes I and II of the respiratory chain and aconitate in the Krebs cycle [32,33], and (ii) by competing reversibly with oxygen for the common binding site on cytochrome c oxidase [34]. On the other hand, attenuation of NO production increases oxygen consumption [35,36]. Since aerobic metabolism is essential in maintaining cardiac function, it is possible that the suppression of mitochondrial respiration by NO may be an important factor affecting ischaemic/reperfusion injury. It may result in a decreased formation of free radical species, which would be beneficial, and it may also lower energy production, which would be detrimental; again, the net effect of these actions remains unknown.

Another possible mechanism by which NO may affect ischaemic/reperfusion injury is via the formation of metabolites such as peroxynitrite, which results from the reaction of NO with superoxide [37,38]. Peroxynitrite may be converted into peroxynitrous acid, which can be cleaved and release an intermediate with hydroxyl radical-like activity [37]. It has been demonstrated that peroxynitrite may exert similar beneficial actions to those of NO, comprising vascular tone and anti-platelet effects [39] and inhibition of leucocyte–endothelium interactions [40]; however, the effect of peroxynitrite in ischaemia/reperfusion has also been controversial, with some studies reporting protection [40,41] and others reporting detrimental effects [14–18].

It is always questionable whether results obtained from animal experimental preparations can be extrapolated to humans. In the present studies, right atrial myocardium, obtained from patients undergoing elective cardiac surgery, was used to investigate the role of NO in ischaemia/reoxygenation injury in humans and to ascertain whether the effect of NO is mediated by respiratory uncoupling or by the generation of radicals such as peroxynitrite.

**METHODS**

**Preparation of atrial slices**

Human right atrial appendages were obtained from the hearts of 34 patients undergoing elective heart surgery. Local ethical committee approval was obtained for the harvesting technique, and the investigation conformed with the principles outlined in the Declaration of Helsinki. Specimens were quickly immersed in cold (4–10 °C) Krebs–Henseleit/Hepes medium, which contained (in mM): NaCl (118), KCl (4.8), NaHCO$_3$ (27.2), KH$_2$PO$_4$ (1), MgCl$_2$ (1.2), CaCl$_2$ (1.25), glucose (10) and Hepes (20). The medium was pre-bubbled with 95% O$_2$/5% CO$_2$ to attain an oxygen partial pressure (P$_{O_2}$) of 25–30 kPa and a pH of 7.4. The atrial appendage was immediately sliced ‘free hand’ with Swann–Morton skin graft blades (Swann-Morton Ltd, Sheffield, U.K.) to a section thickness of 0.3–0.5 mm and a weight of 30–50 mg each, as originally described for the preparation of rat renal slices [42]. Briefly, the tissue was placed with its epicardial side face down on filter paper fixed to a rectangular glass base (5 cm × 25 cm). A ground-glass slide (2.5 cm × 7.5 cm) was then pressed against the tissue and the blade was drawn between the slide and the tissue. The slicing apparatus and the atrium were kept wet at all times with medium cooled on ice (4–10 °C).

**Experimental time course**

After preparation, the slices (between three and five per specimen) were blotted with wet filter paper and loaded into glass conical flasks (25 ml Erlenmeyer flasks; Schott Glaswerke, Mainz, Germany) containing 5 ml of incubation medium and bubbled continuously with 95% O$_2$/5% CO$_2$ to maintain a P$_{O_2}$ of 25–30 kPa and a pH of 7.4. The preparation was then placed in a shaking water-bath (100 cycles/min) at 37 °C for a 30 min equilibration period. Following this, some of the slices were rinsed with the medium, blotted and added to new flasks, which also contained 5 ml of oxygenated medium, for another 240 min, to serve as time-matched aerobic controls. The other slices were first rinsed in medium bubbled with 95% N$_2$/5% CO$_2$ at a pH of 6.8, and then incubated into new flasks containing 5 ml of the same medium, which was bubbled continuously with 95% N$_2$/5% CO$_2$ and maintained at 37 °C for 120 min of simulated ischaemia. Monitoring of P$_{O_2}$ with an oxygen detector electrode (Oxylite*; Optronix Ltd, Oxford, U.K.) revealed that the P$_{O_2}$ in the medium was 0 kPa. In this solution, glucose was removed and replaced with 2-deoxy-D-glucose (grade II; 10 mM). At the end of the ischaemic period, slices were first rinsed in oxygenated medium and

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then incubated further in 5 ml of oxygenated medium at 37 °C with added glucose for another 120 min. All agents to be tested were added during the last 15 min of the equilibration period and then incubated with the slices throughout the entire ischaemic and reoxygenation periods. At the end of the experimental protocols, samples from the incubation media used during the reoxygenation period were collected for the assessment of lactate dehydrogenase (LDH) leakage and the tissue was taken for the assessment of viability [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], oxygen consumption and protein nitrotyrosine content.

We have demonstrated previously that the thickness of the myocardial specimens and the PO₂ of the media used in the present studies are sufficient to maintain the preparation viable beyond 24 h [43], a period clearly longer than the duration of the protocols applied here (< 5 h). It is of note that, because the provision of O₂ and nutrients was achieved by diffusion in our preparation and not arterially, the term ‘reoxygenation’, instead of reperfusion, has been used throughout the paper when describing our results. However, the term reperfusion has been used when referring to results in the literature obtained from experiments in which hearts were arterially perfused following a period of ischaemia.

**Study groups**

The studies were performed in two parts: Study 1 investigated the role of endogenous NO and L-arginine, and Study 2 examined the role of exogenous NO with regard to the injury sustained during ischaemia and reoxygenation.

In Study 1, five groups (n = 6–8 preparations, each from the right atrium of equal numbers of patients per group) were investigated: (i) time-matched aerobic control, (ii) ischaemia/reoxygenation alone, (iii) ischaemia/reoxygenation plus L-arginine (10 mM), (iv) ischaemia/reoxygenation plus D-arginine (10 mM), and (v) ischaemia/reoxygenation plus N⁰-nitro-L-arginine methyl ester (L-NAME; 0.5 mM). In additional experiments, L-NAME was used in combination with L-arginine to investigate whether any beneficial effect induced by L-arginine may be mediated by enhancement of NO production.

In Study 2, the following groups (n = 6–8 preparations, each from the right atrium of equal numbers of patients per group) were investigated: (i) time-matched aerobic control, (ii) ischaemia/reoxygenation alone, and (iii) ischaemia/reoxygenation plus S-nitroso-N-acetylpenicillamine (SNAP) at various concentrations (1, 100, 500 and 1000 μM).

**LDH leakage**

The activity of LDH released into the media, taken as a measure of tissue damage, was assayed by a kinetic UV method based on the formation of NAD⁺ (Sigma catalogue no. 1340-K), and is expressed as units/g wet wt.

**Tissue viability**

The MTT assay was used to quantify tissue viability. In this assay, the yellow MTT is reduced to a blue formazan product by the mitochondria of viable tissue. Briefly, at the end of the experiment, the slices were loaded into a Falcon conical tube (15 ml; Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) and 2 ml of PBS (0.05 M) containing MTT (1.25 mg/ml; 3 mM final concentration) was added. The specimens were incubated for 30 min at 37 °C. Following this, the slices were homogenized in 2 ml of DMSO using an Ultra-Turrax T25 homogenizer equipped with dispersing tool G8 (IKA-Labortechnic, Staufen, Germany) at 9500 rev./min for 1 min. The homogenate was then centrifuged at 1000 g for 10 min. After this, 0.2 ml of supernatant was dispensed into a 98-well microtiter plate (Nunc Brand Products, Roskilde, Denmark) and the absorbance was measured on a plate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 570 nm, and expressed as A/mg wet wt.

**Assessment of protein nitrotyrosine**

Myocardial oxygen consumption

Oxygen consumption by the slices was measured by a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). The electrode contained 1 ml of air-saturated incubation medium (pH 7.4), which was maintained at 37 °C. The slices were loaded carefully into the chamber, avoiding the formation of bubbles. Oxygen consumption was recorded for 5–8 min and was calculated from the decrease in oxygen concentration and expressed as nmol of O₂/g wet wt.

**Statistical analysis**

Data are expressed as means ± S.E.M. of n = 6–8 preparations. One-way ANOVA was used for multiple comparisons. P < 0.05 was considered to be statistically significant.
RESULTS

Effects of endogenously produced NO

(i) LDH leakage
As shown in Figure 1(A), ischaemia/reoxygenation alone induced a significant increase in LDH leakage compared with the time-matched aerobic control group. The incubation of the tissue with l-arginine before and during ischaemia and throughout the reoxygenation period significantly decreased LDH leakage. Preliminary dose–response studies had shown that 10 mM l-arginine was the optimal concentration in our preparation. In contrast, d-arginine and l-NAME had no significant effects. In additional experiments (Figure 2A), incubation of the tissue with l-arginine and l-NAME in combination did not abolish the decrease in LDH leakage seen in the presence of l-arginine alone, suggesting that the effect of l-arginine on LDH leakage is not mediated by the NO pathway. Also in preliminary experiments (results not shown), we had shown that aerobic incubation of tissue with l-arginine, d-arginine, l-NAME or SNAP, at the concentrations used in the present studies, had no effect on the leakage of LDH and the reduction of MTT (see below), which supports the view that the observed decrease in LDH leakage in the presence of l-arginine is a true anti-ischaemic effect.

(ii) MTT reduction
The evaluation of tissue viability by the MTT assay shown in Figure 1(B) demonstrates that ischaemia/reoxygenation significantly decreased the reduction of MTT, by more than 40% compared with the time-matched aerobic control mean values. Interestingly, in contrast with the results for LDH leakage, incubation of tissue with l-arginine, d-arginine or l-NAME alone (Figure 1B), or with l-arginine and l-NAME in combination (Figure 2B), had no effect on MTT reduction.

(iii) Myocardial oxygen consumption
As shown in Figure 1(C), by the end of the reoxygenation period oxygen consumption was markedly decreased, by more than 40% compared with the values for myocardium incubated aerobically. This decrease in oxygen consumption was not significantly affected by incubation of the tissue with l-arginine or l-NAME.

Figure 1 Effects of l-arginine on (A) LDH leakage into the incubation medium, (B) MTT reduction, (C) oxygen consumption and (D) protein nitrotyrosine content in human atrial myocardium during ischaemia/reoxygenation
Myocardium was perfused aerobically, or subjected to 120 min of ischaemia and 120 min of reoxygenation in the presence or absence of l-arginine, d-arginine or l-NAME. Data are expressed as means ± S.E.M. of between six and eight preparations, each from the right atrium of equal numbers of patients. Significance of differences: *P < 0.05 compared with aerobic control group; †P < 0.05 compared with no treatment group. OD denotes absorbance.
Figure 2 Effects of L-arginine and L-NAME on (A) LDH leakage into the incubation medium and (B) MTT reduction in human atrial myocardium during ischaemia/reoxygenation

Myocardium was perfused aerobically, or subjected to 120 min of ischaemia and 120 min of reoxygenation in the presence or absence of L-arginine alone or L-arginine plus L-NAME in combination. Data are expressed as means ± S.E.M. of between six and eight preparations, each from the right atrium of equal numbers of patients. Significance of differences: *P < 0.05 compared with aerobic control group; †P < 0.05 compared with no treatment group.

(iv) Protein nitrotyrosine

As shown in Figure 1(D), the mean protein nitrotyrosine level in the tissue of the aerobic control group was 7.0 ± 1.4 nmol/mg of protein. Unexpectedly, this value was not significantly affected by ischaemia/reoxygenation, or by incubation with a NO precursor and inhibitor.

Effects of exogenous NO

(i) LDH leakage

Figure 3(A) confirms the results of the previous study, in that ischaemia/reoxygenation resulted in significantly greater LDH leakage compared with the values seen for the aerobic control group. However, in contrast with the decrease in LDH leakage induced by L-arginine, no significant effect was observed when sections were incubated with various concentrations of the NO donor SNAP.

(ii) MTT reduction

Figure 3(B) shows that incubation of the tissue with various concentrations of SNAP had no significant effect on MTT reduction when compared with the mean values obtained in the ischaemia/reoxygenation group not receiving the drug.

(iii) Myocardial oxygen consumption

SNAP at two selected concentrations, 1 and 1000 μM, did not modify the decreased oxygen consumption induced by ischaemia/reoxygenation (55.8 ± 5.5% and 52.2 ± 7.7% respectively, compared with 56.1 ± 4.4% for the aerobic control).

(iv) Protein nitrotyrosine

The myocardial protein nitrotyrosine content was not significantly affected by ischaemia/reoxygenation (8.1 ± 1.4 nmol/mg of protein, compared with 7.0 ± 1.5 nmol/mg in the aerobic control group) or by incubation with the NO donor SNAP at concentrations of 1 and 1000 μM (6.9 ± 0.8 and 8.3 ± 1.2 nmol/mg of protein).
DISCUSSION

The present studies have demonstrated for the first time that L-arginine exerts modest, but significant, protection against ischaemic/reoxygenation injury of the human myocardium, and that NO does not play a significant role in this. These findings contrast with the reported protective [1–7] or detrimental [8–13] effects of NO in other species, and add further to the controversy regarding the role of NO in myocardial ischaemic/reperfusion injury. Our results provide new insight into the role of NO during ischaemia and reoxygenation, and have important clinical implications that are discussed below.

The first notable finding of our studies is the anti-ischaemic effect of L-arginine, which is supported by experimental animal studies \textit{in vivo} and with isolated perfused hearts \textit{in vitro} [3,4,8]. However, it should be emphasized that, in contrast with the animal studies, the degree of protection by L-arginine seen for human tissue was modest (e.g. decrease in LDH leakage, but no effects on MTT reduction or myocardial oxygen consumption). There is no general agreement on the effect of L-arginine and, in fact, some animal studies have reported that L-arginine may increase cardiac injury, an effect that can be prevented by inhibiting NO production [10,11]. The reason for these opposing results is unclear, but the type of experimental preparation and conditions, the time of administration of L-arginine (e.g. before or during ischaemia or at reperfusion) and the doses given may be contributory factors. Thus, for example, Kronon et al. [45] have recently reported in studies on neonatal piglets that supplementing the cardioplegic solution with 4 mmol/l L-arginine is cardioprotective, whereas a concentration of 10 mmol/l is detrimental.

The mechanism by which L-arginine exerts its effect also remains unclear, but our studies strongly suggest that the mechanism of action of L-arginine does not involve NO (e.g. protection was not cancelled by L-NAME). This result is consistent with the massive loss of intracellular arginine occurring in hearts following ischaemia/reperfusion, a phenomenon that has been associated with an increase in endothelin-1 levels and with cardiac dysfunction [4], but it contradicts the results obtained in animal studies in which the protective effect of L-arginine was reported to be mediated by NO [3,4,8]. It has also been suggested that arginine-depleted cells generate both superoxide and NO via NOS, the interaction of which may lead to peroxynitrite formation and cellular injury [46–48]. Yet the latter mechanism cannot be considered to explain the results of the present studies, since L-arginine did not affect the formation of peroxynitrite. L-Arginine may still possess metabolic and functional effects that affect ischaemia/reperfusion injury. Our studies, however, have shown that the observed decrease in oxygen consumption as a result of ischaemia/reoxygenation was not affected by L-arginine and that, therefore, it is unlikely that this mechanism may account for the protective effect of L-arginine in our tissue preparation. Furthermore, Weyrich et al. [3] have suggested that the induction of coronary reperfusion by L-arginine is unlikely to be a primary mechanism of its effect of preserving endothelial function and decreasing myocardial injury. Certainly, further investigation is required to elucidate the mechanism of the beneficial effect of L-arginine.

A second important finding of our studies is the absence of a significant role for NO in a model of ischaemic/reoxygenation injury in the human myocardium. Thus neither the inhibition of endogenous NO production by L-NAME nor the exogenous administration of several concentrations of an NO donor influenced tissue viability, LDH leakage, oxygen consumption or the formation of nitrotyrosine. These results were unexpected, since other investigators have reported induction by NO of beneficial [1–7] or detrimental [8–13] effects in quite a narrow range of concentrations, but they question the relevance of NO metabolism in the human myocardium when subjected to ischaemia/reoxygenation. Our studies do not disclose the reason for these differences. A number of studies in the literature have reported the activity of NOS and the production of NO during ischaemia; again these are controversial, with some investigators reporting increased NOS activity [49] and elevated NO release [50], and others observing decreased NOS activity [51,52] and lowered NO release [1,2,7]. Of course, the elucidation of these fundamental facts are central to the understanding of whether and how the manipulation of NO metabolism may affect ischaemia/reperfusion injury.

In trying to find an explanation for the results of the present studies, it may be speculated that our preparation is composed mainly of myocytes, and that O$_2$ and nutrients are provided by diffusion and not through the vasculature, as opposed to other \textit{in vivo} and \textit{in vitro} studies in which the heart was arterially perfused and NO was found to have an effect [1–13]. Because the endothelium is the main source of NO in the heart, it may be argued that any action of NO on the myocardium during ischaemia/reperfusion has to involve the endothelium. Furthermore, since platelets and neutrophils play an important role in ischaemic/reperfusion injury, and since NO inhibits platelet adhesion [23] and aggregation [24] and decreases neutrophil activation [25], our bloodless preparation may be another potential explanation for the lack of effect of NO in the present studies.
A potential limitation of our studies is the use of the right atrial appendages, as opposed ventricular tissue, so that any extrapolation of the results to the latter must be conducted with caution. Nonetheless, Walker et al. [53] have suggested that identical ischaemic injury and protection can be obtained by preconditioning in both tissues. Another possible limitation may be that right atrial appendages were obtained from patients undergoing elective coronary bypass graft surgery, and all the patients were receiving NO donors before the operation. Since NO has been suggested to be involved in the early [54] and late [55] phases of ischaemic preconditioning, it could be argued that the myocardial specimens used in the present studies were already preconditioned, and that this may have masked the effects derived from the manipulation of NO metabolism. However, this is unlikely for our preparation, because of the demonstration in our laboratory [56] that the right atrial myocardium obtained from patients receiving NO donors can be preconditioned by ischaemia.

In conclusion, our studies show that t-arginine affords modest protection against ischaemic/reoxygenation injury in a quiescent model of the human myocardium, an action that is NO-independent, and that the NO metabolism does not play a significant role. The results may have clinical implications, but caution should be exercised when extrapolating results obtained in an in vitro preparation to the in vivo setting.

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