British Journal of Pharmacology (1998) 123, 1159–1167

Effect of nitrovasodilators and inhibitors of nitric oxide synthase on ischaemic and reperfusion function of rat isolated hearts

Eugene F. du Toit, Joy McCarthy, Jody Miyashiro, Lionel H. Opie & [1,2]Friedrich Brunner

Ischaemic Heart Disease Research Unit, University of Cape Town Medical School, Cape Town 7925, South Africa and [1]Institut für Pharmakologie und Toxikologie, Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria

1 The functional role of the nitric oxide (NO)/guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway in experimental myocardial ischaemia and reperfusion was studied in rat isolated hearts.

2 Rat isolated hearts were perfused at constant pressure with Krebs-Henseleit buffer for 25 min (baseline), then made ischaemic by reducing coronary flow to 0.2 ml min^{-1} for 25 or 40 min, and reperfused at constant pressure for 25 min. Drugs inhibiting or stimulating the NO/cyclic GMP pathway were infused during the ischaemic phase only. Ischaemic contracture, myocardial cyclic GMP and cyclic AMP levels during ischaemia, and recovery of reperfusion mechanical function were monitored.

3 At baseline, heart rate was 287 ± 12 beats min^{-1}, coronary flow was 12.8 ± 0.6 ml min^{-1}, left ventricular developed pressure (LVDevP) was 105 ± 4 mmHg and left ventricular end-diastolic pressure 4.6 ± 0.2 mmHg in vehicle-treated hearts (control; $n = 12$). Baseline values were similar in all treatment groups ($P > 0.05$).

4 In normoxic perfused hearts, 1 μM N^G-nitro-L-arginine (L-NOARG) significantly reduced coronary flow from 13.5 ± 0.2 to 12.1 ± 0.1 ml min^{-1} (10%) and LVDevP from 97 ± 1 to 92 ± 1 mmHg (5%; $P < 0.05$, $n = 5$).

5 Ischaemic contracture was 46 ± 2 mmHg, i.e. 44% of LVDevP in control hearts ($n = 12$), unaffected by low concentrations of nitroprusside (1 and 10 μM) but reduced to ~ 30 mmHg ($\sim 25\%$) at higher concentrations (100 or 1000 μM; $P < 0.05$ vs control, $n = 6$). Conversely, the NO synthase inhibitor L-NOARG reduced contracture at 1 μM to 26 ± 3 mmHg (23%), but increased it to 63 ± 4 mmHg (59%) at 1000 μM ($n = 6$). Dobutamine (10 μM) exacerbated ischaemic contracture (81 ± 3 mmHg; $n = 7$) and the cyclic GMP analogue Sp-8-(4-p-chlorophenylthio)-3′,5′-monophosphorothioate (Sp-8-pCPT-cGMPS; 10 μM) blocked this effect (63 ± 1 mmHg; $P < 0.05$ vs dobutamine alone, $n = 5$).

6 At the end of reperfusion, LVDevP was 58 ± 5 mmHg, i.e. 55% of pre-ischaemic value in control hearts, significantly increased to $\sim 80\%$ by high concentrations of nitroprusside (100 or 1000 μM) or L-NOARG at 1 μM, while a high concentration of L-NOARG (1000 μM) reduced LVDevP to $\sim 35\%$ ($P < 0.05$ vs control; $n = 6$).

7 Ischaemia increased tissue cyclic GMP levels 1.8 fold in control hearts ($P < 0.05$; $n = 12$); nitroprusside at 1 μM had no sustained effect, but increased cyclic GMP ~ 6 fold at 1000 μM; L-NOARG (1 or 1000 μM) was without effect ($n = 6$). Nitroprusside (1 or 1000 μM) marginally increased cyclic AMP levels whereas NO synthase inhibitors had no effect ($n = 6$).

8 In conclusion, the cardioprotective effect of NO donors, but not of low concentrations of NO synthase inhibitors may be due to their ability to elevate cyclic GMP levels. Because myocardial cyclic GMP levels were not affected by low concentrations of NO synthase inhibitors, their beneficial effect on ischaemic and reperfusion function is probably not accompanied by reduced formation of NO and peroxynitrite in this model.

Keywords: Nitric oxide; nitric oxide synthase inhibitor; ischaemic contracture; reperfusion injury; rat isolated perfused heart

Introduction

Nitrovasodilators have been used as therapeutic agents for the treatment of angina pectoris for more than a century. These drugs release nitric oxide (NO) which activates soluble guanylyl cyclase resulting in stimulated production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and vasorelaxation (Waldman & Murad, 1987). NO is also formed endogenously by NO synthase of coronary endothelium and appears to regulate coronary tone both in experimental animals and man. Although myocytes may also synthesize NO under certain conditions, neither the regulation of this pathway nor the functions of myocyte-derived NO are well understood (Kelly *et al*., 1996).

Myocardial ischaemia and reperfusion are associated with loss of active coronary dilatation, impairment of myocardial mechanical function and cardiac arrhythmias (Opie, 1989). The role of NO in this phenomenon of reperfusion injury has been studied intensively, but it is still unclear whether NO is protective or injurious to the reperfused heart (for recent review see Curtis & Pabla, 1997). In the majority of studies, reperfusion injury has been attributed to a reduced formation or activity of NO because the NO precursor L-arginine (Weyrich *et al*., 1992; Yang 1993; Sato *et al*., 1995), several NO donors (Johnson *et al*., 1990; Siegfried *et al*., 1992; Lefer *et al*., 1993), interventions resulting in stimulated NO production involving endothelial receptors (Linz *et al*., 1992; Richard *et al*., 1994) and lipopolysacharide (Yang *et al*., 1997) have all been found to protect the heart. Such findings prompted the suggestion that surgical myocardial reperfusion injury might

[2] Author for correspondence.

best be avoided with NO replacement therapy (Lefer, 1995). However, in some studies blockade of NO synthase with N^G-nitro-L-arginine (L-NOARG) or N^G-nitro-L-arginine methyl ester (L-NAME) was without effect on myocardial mechanical function (Woditsch & Schrör, 1992; Pabla & Curtis, 1996), and in several instances, these blockers even improved reperfusion function (Matheis *et al*., 1992; Schulz & Wambolt, 1995; Wang & Zweier, 1996). In the latter studies, protection was attributed to a reduced formation of peroxynitrite (Yasmin *et al*., 1997) which is generally considered to be injurious to tissues.

To compare these studies is not easy, since hearts were from different animal species, perfusion was done *in vitro* or *in vivo*, the duration and type of ischaemia was different, and because vastly different experimental protocols involving application of test drugs from before ischaemia into reperfusion, during ischaemia and part of reperfusion, or during ischaemia alone were used. Therefore, the aim of the present study was to assess the effects of NO donors and NO synthase inhibitors, used at low and high concentrations and reliably administered from the beginning to the end of the ischaemic period, on ischaemic contracture and reperfusion function in one and the same model. We hypothesized that activation of the myocardial NO/cyclic GMP pathway would attenuate myocardial injury by increasing tissue cyclic GMP levels (Pabla & Curtis, 1995) and that inhibition of the pathway would exacerbate myocardial injury. Since inconsistent changes in cyclic GMP (and cyclic AMP) have been found after ischaemia and reperfusion in rat isolated hearts (Pabla *et al*., 1995) and anaesthetized animals (Kane *et al*., 1985; Barnes & Coker, 1995), the effect of ischaemia on myocardial levels of these mediators was also determined and compared to heart function.

Methods

Heart perfusion

Male Long-Evans rats weighing 250–300 g were anaesthetized with diethyl ether, given an intravenous injection of 200 iu heparin, and hearts were excised and placed in ice-cold Krebs-Henseleit perfusion buffer before being mounted on a Langendorff apparatus for perfusion at 37°C with Krebs-Henseleit buffer at constant pressure (100 cmH_2O). The buffer was equilibrated with 95% CO_2/5% O_2 and had the following composition (mM): NaCl 118.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25 and KH_2PO_4 1.2). Retrograde perfusion was resumed within 45 s of excision of the heart. A 'cling-film' balloon (Curtis *et al*., 1986) was inserted into the left ventricle and attached to a pressure transducer. Vehicle or test compounds were infused directly above the heart. Cardiac parameters were monitored continuously and included heart rate, left-ventricular developed pressure (LVDevP; difference between left-ventricular peak systolic pressure and end-diastolic pressure), and coronary flow obtained from timed collections of coronary effluent or with an electromagnetic flowmeter (Narcomatic RT 500, Narco Bio-Systems). All animals received humane care in accordance with the Principles of Laboratory Animal Care of the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (NIH publication no 80-23, revised 1978).

Experimental protocols

Effects of NO donors and NO synthase inhibitors in normoxic hearts Hearts were equilibrated and baseline parameters determined (30 min). Then, increasing concentrations of nitroprusside (1–1000 μM) or L-NOARG (0.01 μM–1 mM) were added to the perfusate, each concentration over 8 min, followed by perfusion without drug (vehicle) for another 8 min (total duration of experiment: 126 min for L-NOARG), and heart rate, coronary flow and LVDevP were determined.

Effects on ischaemic contracture Hearts were equilibrated, followed by perfusion at 0.2 ml min^{-1} for 40 min at 37°C. Amplitude of ischaemic contracture (mmHg) was assessed in the presence of vehicle or one of the following drugs which were infused during the ischaemic phase only: nitroprusside (1, 10, 100 and 1000 μM), S-nitroso-N-acetyl-DL-penicillamine (SNAP; 100 μM), N^G-nitro-L-arginine (L-NOARG; 0.1, 1, 10 and 1000 μM), L-NAME (0.1, 1 and 10 μM), dobutamine (10 μM), the combination of dobutamine (10 μM) and Sp-8-(4-p-chlorophenylthio)-3′,5′-monophosphorothioate (Sp-8-pCPT-cGMPS; 10 μM) (Butt *et al*., 1994), or dobutamine (10 μM) and 8-bromo cyclic GMP (100 μM).

Effects on ischaemic tissue cyclic nucleotide levels In a second series of experiments, hearts were equilibrated and coronary flow was reduced to 0.2 ml min^{-1} as above and test compounds were added, starting from the beginning of ischaemia, over 5, 15 or 25 min. At these times hearts were freeze-clamped for the determination of tissue cyclic AMP, cyclic GMP, ATP and creatine phosphate.

Effects on mechanical function during reperfusion After equilibration (30 min) hearts were perfused at 0.2 ml min^{-1} for 25 min (low-flow ischaemia), and drugs which had affected ischaemic contracture were added to the perfusate during these 25 min (i.e., the ischaemic phase). The hearts were then reperfused without drugs. The following drugs were tested: nitroprusside (1, 10, 100 and 1000 μM), SNAP (100 μM), L-NOARG (1 and 1000 μM), L-NAME (10 μM) and dobutamine (10 μM). In these experiments, reperfusion function was documented. To establish whether the NO synthase inhibitors influenced free-radical induced injury, we perfused two groups of hearts during ischaemia with superoxide dismutase (EC 1.15.1.1; 10^{-5} iu L^{-1}) together with catalase (EC 1.11.1.6; 10^{-6} iu L^{-1}). To establish whether L-NOARG afforded additional protection, L-NOARG (1 μM) was infused together with superoxide dismutase and catalase.

Biochemical analyses Myocardial tissue ATP and creatine phosphate levels were determined by standard enzymatic methods (Lamprecht *et al*., 1974; Lamprecht & Trautschold, 1974). Cyclic nucleotides were determined by radioimmunoassay with our own antibody for cyclic GMP (Kukovetz *et al*., 1979) and a commercial kit for cyclic AMP (Amersham, U.K.). Briefly, hearts were freeze-clamped, lyophilized and 10–15 mg of dry ventricular tissue was extracted in 5% trichloroacetic acid (for determination of cyclic GMP) or perchloric acid (for determination of cyclic AMP). Trichloroacetic acid was removed by diethyl ether extraction (3 washes of 5 min; 3 fold excess), the aqueous extracts were acetylated resulting in a ten fold increase in sensitivity (Harper & Brooker, 1975) and appropriately diluted for determination of cyclic GMP by radioimmunoassay (IC_{50} of standard curve: ~20–25 fmol per tube). Aqueous samples containing cyclic AMP were neutralized with KOH and subjected to radioimmunoassay without acetylation of nucleotide (IC_{50} of standard curve: ~2 nmol per tube).

Drugs

Dobutamine hydrochloride was obtained from Eli Lilly Pharmaceuticals (Cape Town, South Africa). Nitroprusside natrium, SNAP, L-NOARG hydrochloride, L-NAME, 8-bromo-cyclic GMP, superoxide dismutase and catalase were obtained from Sigma Chemicals Company (Midrand, South Africa). Compounds were dissolved in de-ionized water and made up on the day of the experiment.

Data analysis

All results are expressed as the means ± s.e.mean, as untransformed values, i.e. heart rate is given as beats min^{-1}, the various pressures as mmHg and coronary flow as ml min^{-1} (the slightly different basal flow rates due to small variations in heart weight were not corrected). Peak ischaemic contracture refers to the peak diastolic pressure of the asystolic heart (King *et al*., 1995) and recovery of ventricular function was taken as the LVDevP at 5, 10, 15, 20 and 25 min of reperfusion. For multiple comparisons ANOVA followed by the Bonferroni correction was applied. A P value of less than 0.05 was considered significant.

Results

Baseline data and effect of drugs on vascular and mechanical function in normoxic hearts

Basal haemodynamic parameters after 30 min perfusion, i.e. just before the onset of ischaemia, are summarized in Table 1. There were no significant differences between groups ($P>0.05$). The effects of L-NOARG (0.1 μM – 1 mM) on vascular and myocardial function in normoxic perfused hearts are shown in Figure 1. Coronary flow and LVDevP were reduced in a concentration-dependent fashion: at 1 μM L-NOARG the reductions were 10% and 5%, and at 1 mM 35% and 66% of control, respectively ($P<0.05$ for both parameters, $n=5$). Heart rate and LVEDP were unchanged at all concentrations of L-NOARG (295 ± 6 beats min^{-1} and 0 mmHg, respectively; not shown). Nitroprusside was without significant effect on coronary flow or LVDevP ($n=5$, not shown).

Effect of NO donors, NO synthase inhibitors and cyclic nucleotide-dependent agents on magnitude of ischaemic contracture

The effects of agents affecting the NO/cyclic GMP pathway on the magnitude of ischaemic contracture are shown in Figure 2 (for number of experiments, see Table 1). Ischaemic contracture of hearts perfused with vehicle was 46 ± 2 mmHg (control). The lower concentrations of nitroprusside (1, 10 μM) were ineffective, whereas 100 and 1000 μM nitroprusside reduced contracture. SNAP (100 μM) was similarly effective (29 ± 3 mmHg; $P<0.05$, not shown). Of the NO synthase inhibitors, the lowest concentration of L-NOARG (0.1 μM) had no effect, 1 μM L-NOARG decreased ischaemic contracture while higher concentrations of the inhibitor tended to have deleterious effects ($P<0.05$ vs control at 1000 μM). The effect of L-NAME (0.1, 1 and 10 μM) showed the same pattern. Figure 3 shows the effect of dobutamine (10 μM), the cardiac action of which involves cellular elevation of cyclic AMP. Dobutamine increased ischaemic contracture and Sp-8-pCPT-cGMPS (10 μM) antagonized this effect ($P<0.05$ vs dobutamine alone); 8-bromo cyclic GMP (10 μM) was without effect ($P>0.05$ vs dobutamine alone; for number of experiments see Table 1).

The levels of the high energy phosphates adenosine 5′-triphosphate (ATP) and creatine phosphate for selected concentrations of nitroprusside and L-NOARG are given in Table 2. There were no differences in the rate of decline in tissue ATP levels between treatment groups and control during the 25 min of ischaemia. As expected, the creatine phosphate level dropped quickly after initiation of ischaemia ($P<0.05$ vs pre-ischaemic control) and was similar at 5, 15 and 25 min of ischaemia.

Effect of NO donors and NO synthase inhibitors on reperfusion mechanical function

The effect of nitroprusside and SNAP on recovery of reperfusion mechanical function is shown in Figure 4. In control hearts mean recovery of LVDevP was 58 ± 5 mmHg (55% of pre-ischaemic level) after 25 min of reperfusion. The low concentration of nitroprusside (1 μM) had no effect while 100 and 1000 μM of the donor increased recovery 2.1 and 2.0

Table 1 Baseline haemodynamic parameters

Drug(s) (μM)	*Number of hearts*	*Heart rate* (beats min^{-1})	*LVEDP* (mmHg)	*LVDevP* (mmHg)	*Coronary flow* (ml min^{-1})
Control (Vehicle)	12	287 ± 12	4.6 ± 0.2	105 ± 4	12.8 ± 0.6
Nitroprusside (1.0)	6	292 ± 8	3.8 ± 0.2	112 ± 3	13.6 ± 0.5
Nitroprusside (10)	6	293 ± 6	4.0 ± 0.3	108 ± 2	12.1 ± 1.0
Nitroprusside (100)	6	293 ± 8	4.3 ± 0.2	114 ± 6	12.1 ± 0.8
Nitroprusside (1000)	6	289 ± 9	4.0 ± 0.2	111 ± 3	12.2 ± 0.7
L-NOARG (0.1)	7	277 ± 10	4.1 ± 0.3	112 ± 2	12.2 ± 0.6
L-NOARG (1.0)	6	282 ± 11	3.8 ± 0.2	112 ± 3	12.6 ± 0.8
L-NOARG (10)	9	293 ± 7	4.5 ± 0.2	104 ± 2	10.4 ± 0.3
L-NOARG (1000)	6	288 ± 7	4.0 ± 0.2	107 ± 3	10.9 ± 0.6
L-NAME (0.1)	6	279 ± 7	4.0 ± 0.1	118 ± 6	11.3 ± 1.4
L-NAME (1.0)	6	299 ± 17	4.2 ± 0.8	104 ± 1	11.6 ± 0.5
L-NAME (10)	6	277 ± 17	4.0 ± 0.6	115 ± 2	12.6 ± 0.5
Dobutamine (10)	7	279 ± 3	4.1 ± 0.5	108 ± 4	13.5 ± 0.7
Dobut + Sp-cGMPS (10)	5	302 ± 12	4.7 ± 0.1	109 ± 2	13.2 ± 0.4
Dobut + 8-Br-cGMP (100)	7	265 ± 13	3.9 ± 0.5	106 ± 4	13.6 ± 0.8

Parameters were determined at end of equilibration (30 min) in normoxic hearts. Values are mean ± s.e.mean of the number of hearts given. LVEDP left ventricular end-diastolic pressure; LVDevP, left ventricular developed pressure; dobut, dobutamine; Sp-cGMPS, Sp-8-pCPT-cGMPS.

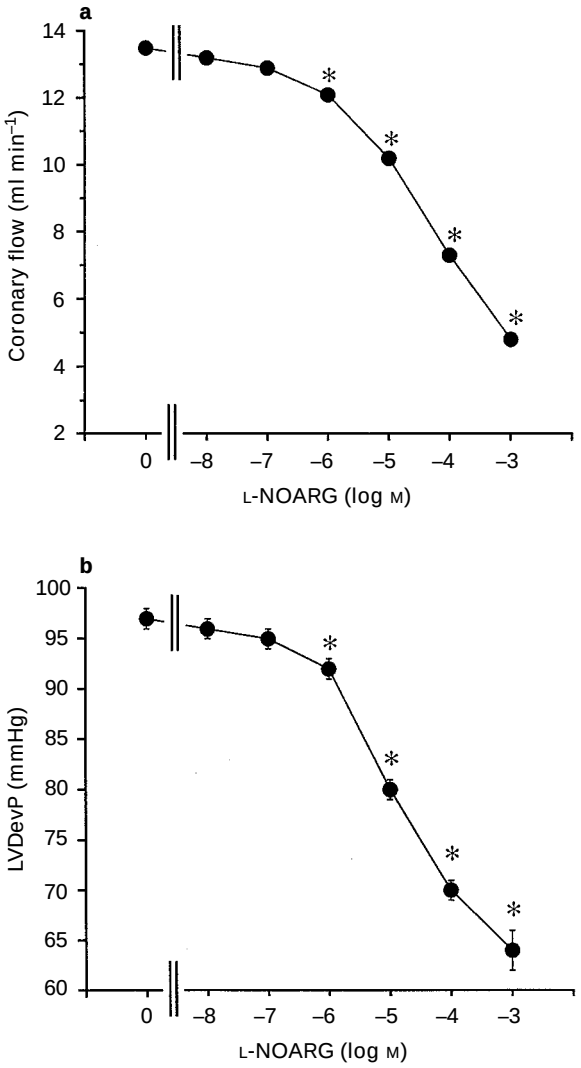

Figure 1 Effect of L-NOARG on coronary flow (a) and LVDevP (b) in normoxic perfused hearts. Hearts were perfused with inhibitor in cumulative fashion, 8 min being allowed for each concentration followed by 8 min of perfusion without drug. Values are mean of 5 hearts; vertical lines show s.e.mean. *$P<0.05$ vs vehicle.

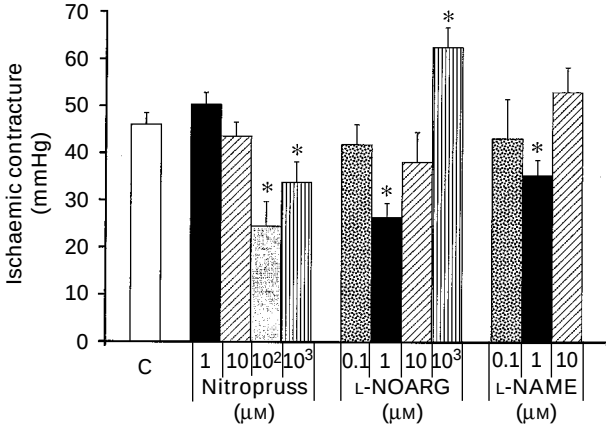

Figure 2 Effect of nitroprusside (nitropruss; 1–1000 μM), L-NOARG (0.1–1000 μM) and L-NAME (0.1–10 μM) on magnitude of peak ischaemic contracture reached during 25 min of ischaemia. Ischaemic contracture is the peak diastolic pressure (mmHg) of the asystolic heart. Baseline LVDevP in control hearts (C) was 105 ± 4 mmHg. Values are mean $\pm$ s.e.mean of 12 (Control) or 6–9 hearts (drug-treated). *$P<0.05$ vs control contracture.

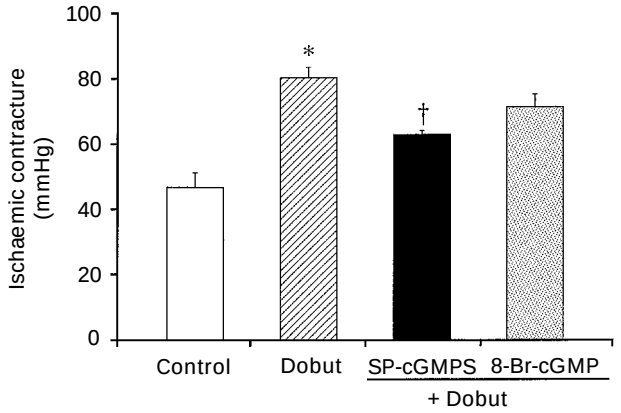

Figure 3 Effect of dobutamine (Dobut, 10 μM) alone and in the presence of the cyclic GMP mimetics Sp-8-(4-p-chlorophenylthio)-3′,5′-monophosphorothioate (Sp-cGMPS; 10 μM) or 8-bromo cyclic (8-Br-cGMP, 100 μM) on the magnitude of the peak ischaemic contracture reached during 25 min of ischaemia. Values are mean $\pm$ s.e.mean of 12 (control) or 5–7 hearts (drugs). *$P<0.05$ vs control contracture; †$P<0.05$ vs dobutamine-induced contracture.

fold, respectively, and SNAP (100 μM) 1.8 fold ($P<0.05$ vs control; $n=6$ in each case).

The effect of inhibitors of NO synthase on reperfusion mechanical function is shown in Figure 5. The low concentration of L-NOARG (1 μM) improved recovery of LVDevP (1.9 fold; $P<0.05$) while the high concentration (1000 μM), which caused prolonged vasoconstriction during reperfusion, reduced LVDevP towards the end of reperfusion by 33%. Treatment with L-NAME (10 μM) during ischaemia ameliorated mechanical function to the same extent as 1 μM L-NOARG ($P<0.05$). In the presence of superoxide dismutase together with catalase, recovery of LVDevP was promptly and uniformly increased (2.1 fold; $P<0.05$ vs vehicle), but no further increase was noted when L-NOARG was co-infused with superoxide dismutase and catalase ($n=6$ in each case). Dobutamine (10 μM) given during ischaemia resulted in a lower recovery of reperfusion LVDevP at 5 and 15 min, but later approached the value for control (55 ± 7 mmHg, $P>0.05$, $n=6$; data not shown).

Effect of NO donors and NO synthase inhibitors on cyclic nucleotide levels

The effect of test drugs on levels of myocardial cyclic GMP during ischaemia are presented in Figure 6. With increasing duration of ischaemia, cyclic GMP levels increased from 22 ± 1 (baseline) to 37 ± 1 pmol g^{-1} over 25 min ($P<0.05$; $n=12$). Tissue cyclic GMP levels were markedly elevated by nitroprusside and SNAP. A low concentration of nitroprusside (1 μM) increased cyclic GMP ~5 fold within 5 min of ischaemia, but this effect had waned after 15 min. At 1000 μM, the effect was more pronounced and sustained (~6 fold above control; $P<0.05$). SNAP (100 μM) was even more potent (~10 fold increase over control). The NO synthase inhibitor L-NOARG (1 or 1000 μM) did not prevent the ischaemia-induced slow increase in cyclic GMP levels ($P>0.05$ compared to control hearts at each time point; $n=6$).

The effect of a low and high concentration (1 and 200 μM) of L-NOARG on the concentration of cyclic GMP in coronary effluent during ischaemia and reperfusion is shown in Figure 7. Release was unaffected by the low concentration and reduced both during the ischaemic and reperfusion phases by the high concentration ($P<0.05$; $n=5$).

Table 2 Levels of high energy phosphates for control hearts and hearts treated with nitroprusside or L-NOARG

Treatment (μM)	*High energy phosphate* (μmol g^{-1} w.w.)	*Pre-ischaemic control*	*Duration of ischaemia* (min) 5	15	25
Control	ATP	3.80±0.26	3.40±0.09	2.51±0.19	1.65±0.23
	PCr	14.0±1.19	1.50±0.30	1.32±0.14	1.42±0.37
Nitroprusside (1.0)	ATP	3.80±0.26	3.06±0.10	2.57±0.04	1.54±0.16
	PCr	14.0±1.19	2.02±0.14	1.51±0.06	1.25±0.05
Nitroprusside (1000)	ATP	3.80±0.26	3.10±0.16	2.02±0.30	1.63±0.08
	PCr	14.0±1.19	1.30±0.22	1.53±0.26	1.37±0.11
L-NOARG (1.0)	ATP	3.80±0.26	3.22±0.26	2.69±0.34	1.82±0.47
	PCr	14.0±1.19	0.92±0.11	1.50±0.12	1.56±0.41
L-NOARG (1000)	ATP	3.80±0.26	3.46±0.38	2.34±0.28	1.58±0.64
	PCr	14.0±1.19	1.44±0.19	1.12±0.26	1.07±0.27

Values of adenosine trisphosphate (ATP) and phosphocreatine (PCr) are expressed as means±s.e.mean of 6 experiments in each case. No significant differences between 5, 15 and 25 min of ischaemia were observed ($P>0.05$). The significant drop in PCr after onset of ischaemia is not indicated.

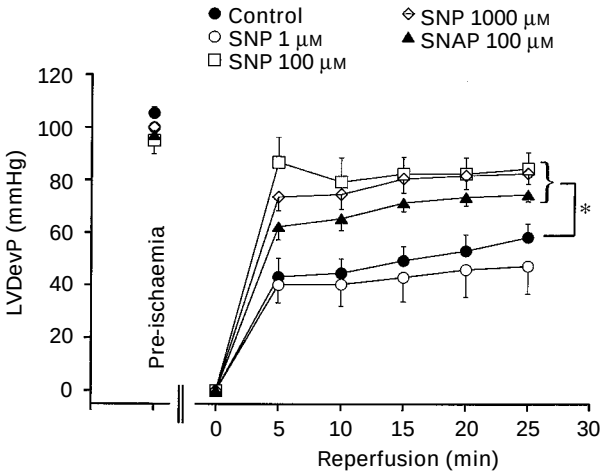

Figure 4 Effect of NO donors on recovery of LVDevP during 25 min of reperfusion. Hearts were perfused with vehicle (Control), nitroprusside (1 μM, 100 μM or 1000 μM) or S-nitroso-N-acetyl-DL-penicillamine (SNAP; 100 μM) during the ischaemic phase only. Values are mean of 6 hearts; vertical lines show s.e.mean $*P<0.05$ vs control (nitroprusside at 1 μM had no significant effect).

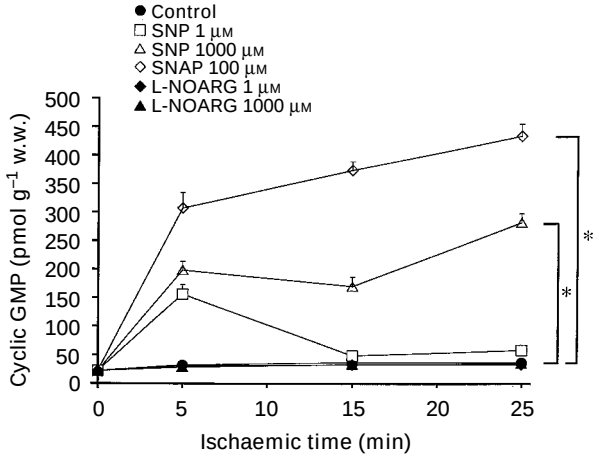

Figure 6 Levels of cyclic GMP in the ventricles of hearts treated with vehicle (Control), nitroprusside (1 μM or 1000 μM), SNAP (100 μM), or L-NOARG (1 μM or 1000 μM). Values are mean of 12 (Control) of 6 hearts (all other conditions); vertical lines show s.e.mean. $*P<0.05$ vs control at 5, 15 or 25 min (the significant increase in cyclic GMP due to ischaemia in vehicle hearts is not indicated separately).

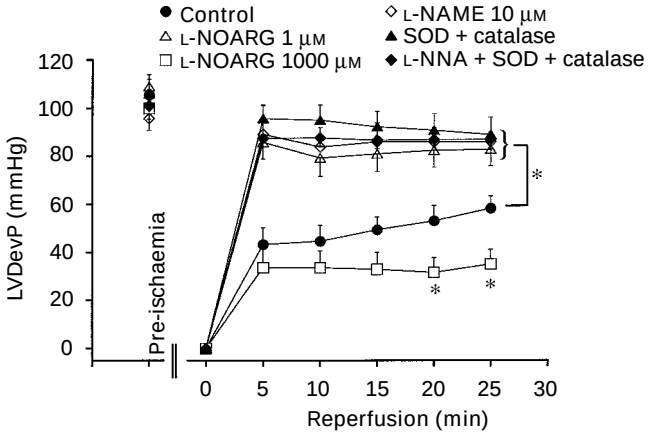

Figure 5 Effect of NO synthase inhibitors and anti-oxidants on recovery of LVDevP during 25 min of reperfusion. Hearts were perfused with vehicle (Control), L-NOARG (1 μM or 1000 μM), L-NAME (10 μM), superoxide dismutase (10^{-5} iu l^{-1}) together with catalase (10^{-6} iu l^{-1}), or L-NOARG (1 μM) together with superoxide dismutase (10^{-5} iu l^{-1}) and catalase (10^{-6} iu l^{-1}) during the ischaemic phase only. Values are mean of 6 hearts; vertical lines show s.e.mean. $*P<0.05$ vs control (L-NOARG at 1 μM had no significant effect up to 15 min of reperfusion).

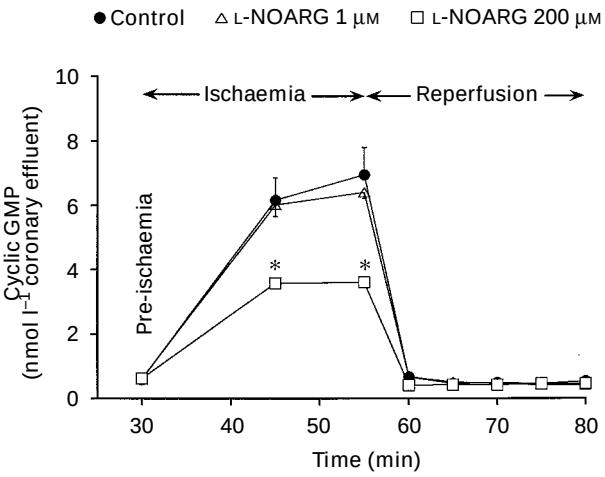

Figure 7 Release of cyclic GMP in coronary effluents during ischaemia and reperfusion in control hearts and hearts treated with L-NOARG at 1 μM or 200 μM. Only the high concentration of L-NOARG reduced cyclic GMP release in ischaemia and reperfusion. Values are mean of 5 hearts; vertical lines show s.e.mean. $*P<0.05$ vs control (the significant reduction during the reperfusion phase is not indicated by asterisks).

The levels of myocardial cyclic AMP are presented in Figure 8. Both a low (1 μM) and high (1000 μM) concentration of nitroprusside increased cyclic AMP at 5 and 25 min ($\sim$1.4 fold and $\sim$1.5 fold, respectively; $P<0.05$ vs control). L-NOARG, whether used at 1 or 1000 μM, had no effect on ischaemic tissue cyclic AMP levels ($P>0.05$ vs control at each time point; $n=6$). The addition of dobutamine (10 μM) during ischaemia elevated tissue cyclic AMP levels $\sim$1.7 fold (means of 6 hearts; data not shown).

Discussion

Although in the majority of the previous studies NO donors protected the heart from experimental ischaemic or reperfusion injury, in several studies inhibitors of NO synthase were found to be protective. We found that NO donors were cardioprotective in millimolar concentrations while they had no effect at lower concentrations. NO synthase inhibitors, on the other hand, were deleterious in millimolar concentrations where they had potent and prolonged vasoconstrictive properties, but were cardioprotective at micromolar concentrations. While NO donors increased tissue cyclic GMP levels, the cardioprotective concentrations of NO synthase inhibitors had no effect on the ischaemic tissue cyclic GMP levels in our model.

Ischaemic contracture was attenuated and recovery of mechanical function during reperfusion was improved in a concentration-dependent fashion by nitroprusside, and the improvement was accompanied by a significant increase in cyclic GMP content of total heart homogenate. Similarly, in a previous study (Pabla & Curtis, 1996), L-NAME hastened the onset of ischaemic contracture and increased its peak, and these effects were prevented by co-perfusion with L-arginine. Several explanations need to be considered. First, there is emerging evidence that endogenous NO is an important inhibitory regulator of cardiac actions of adrenoceptor agonists (Ebihara & Karmazyn, 1996), probably through a mechanism resulting in a reduced Ca^{2+} current in cardiomyocytes (Méry *et al*., 1993; Wahler & Dollinger, 1995). The anti-adrenergic effects of NO may decrease cytosolic Ca^{2+} levels during ischaemia and prevent or decrease cytosolic Ca^{2+} overload during reperfusion. In support, ischaemic contracture is inhibited by nisoldipine, a L-type Ca^{2+} channel antagonist

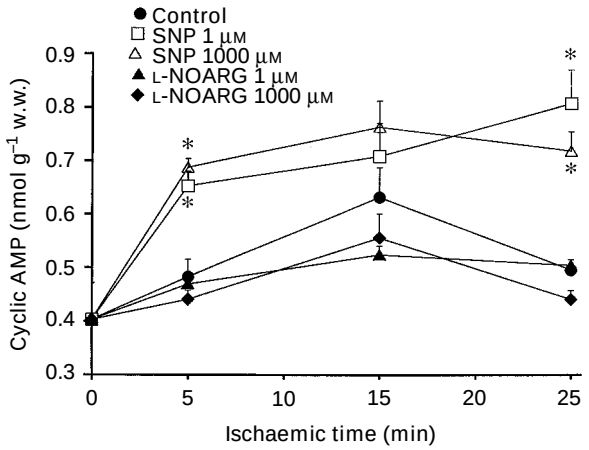

Figure 8 Levels of cyclic AMP in ventricle of hearts treated with vehicle (Control), nitroprusside (1 μM or 1000 μM), or L-NOARG at 1 μM or 1000 μM. Values are mean of 12 (Control) or 5 hearts (all other conditions); vertical lines show s.e.mean. *$P<0.05$ vs control at the respective times. L-NOARG had no significant effect ($P>0.05$).

(Saida *et al*., 1994). In the present study nitroprusside stimulated the synthesis of cyclic GMP, which probably counteracted the pro-ischaemic and energy-expending actions of endogenous noradrenaline. The contracture-lessening effect of Sp-8-pCPT-cGMPS following its exacerbation with dobutamine (Figure 3) also agrees with this view (the reason for the lack of a significant effect of 8-bromo cyclic GMP at the dose used is unclear). Secondly, the beneficial effects of NO donors might result from their anti-endothelin actions (Brunner *et al*, 1995), possibly by attenuating the elevation of intracellular Ca^{2+} concentrations (Ebihara *et al*., 1996). Thirdly, in non-physiologically high concentrations NO may decrease ischaemic contracture and reperfusion injury by virtue of its negative inotropic properties, associated with myofilament desensitization to Ca^{2+} mediated by cyclic GMP-dependent protein kinase (Shah *et al*., 1994). The better ATP preservation during ischaemia is thought to be due to decreased energy demands associated with myofilament desensitization and decreased contractile activity during ischaemia and reperfusion (Vanoverschelde *et al*., 1994). Our findings (Table 2) indicate that tissue high energy phosphate levels were similar on control and NO donor-treated hearts, which may exclude high energy phosphate preservation as a possible cardioprotective mechanism for the donors used in this study. Fourthly, NO donors may also act to preserve endothelial integrity, possibly by an anti-superoxide action (Siegfried *et al*., 1992). Finally, inorganic ($NaNO_2$) and organic NO donors were previously found to decrease polymorphonuclear leucocyte adherence and infiltration of microvascular endothelium as well as attendant tissue injury in rat perfused hearts (Pabla *et al*., 1996), open-chest dogs (Lefer *et al*., 1993) and cats (Weyrich *et al*., 1992). Together with a previous study (Pabla & Curtis, 1996), the present results show that endogenous NO protects rat hearts from reperfusion injury via a polymorphonuclear cell-independent mechanism. Additional details on the role of NO in myocardial ischaemia/reperfusion are summarized in a recent review (Lefer & Lefer, 1996).

Recovery of mechanical function during reperfusion was improved by low concentrations of L-NOARG and L-NAME and inhibited by a high concentration of L-NOARG (Figure 5), although neither the high nor the low concentration of the NO synthase inhibitors significantly affected the ischaemia-induced rise in cyclic GMP content of total heart homogenate. While the deterioration of functional recovery in the presence of 1000 μM L-NOARG mirrors the improvement observed with NO donors discussed above, the cardioprotective mechanism for NO synthase inhibitors used at low concentrations needs consideration. The question of whether NO generation was affected by the low (cardioprotective) concentrations of inhibitors, and the mechanism of protection need to be examined. We first tested the effect of low and high concentrations of L-NOARG in normoxic hearts (Figure 1) to determine any direct effects this inhibitor may have on heart function. The effect of L-NOARG on coronary flow and LVDevP was concentration-dependent and statistically significant at inhibitor concentration of 1 μM or higher. The reduction in LVDevP seen in these hearts (Figure 1b) was probably the effect of limiting coronary flow, as shown previously in a model of constant flow perfusion in which LVDevP was unaffected (Pfeiffer *et al*., 1996). The exact concentration necessary for complete inhibition of NO synthase cannot be determined from these experiments, but appears to be close to 1 mM. In the rat mesentery, acetylcholine-induced vasodilatation was completely inhibited by 200 μM L-NOARG (Moore *et al*., 1990). Although not

measured, L-NOARG is expected to have inhibited NO production in similar fashion after ischaemia and the vasoconstriction found with the highest inhibitor concentrations is likely to have reduced contractile recovery in reperfused hearts independent of ischaemia. Taken together, the considerable myocardial protection in the presence of low concentrations of NO synthase inhibitor during ischaemia is inferred to have occurred despite some inhibition of the enzyme.

In the present study we also found that low doses of NO synthase inhibitors were without effect on the levels of cyclic GMP in myocardial tissue or coronary effluent, suggesting that a mechanism independent of NO synthase might be involved. Indeed, one group has suggested that non-vasoactive concentrations of NO synthase inhibitors protect the heart against ischaemic damage by stimulation of glycolysis from exogenous glucose (Depré *et al*., 1995). However, we were unable to show any energy sparing effects, as reflected by myocardial high energy phosphate levels, in our study. The absence of differences in ATP and creatine phosphate levels between control and inhibitor-treated hearts in our study may be due to the better preservation of ATP seen by us under control conditions. Depré and coworkers obtained control (i.e., vehicle-treated) high energy phosphate levels which were far lower than those seen in our study with the rat heart model.

The hypothesis of a NO-independent action of low concentrations of NO synthase inhibitors appears strengthened, because both inhibitors were found to be protective at lower concentrations than those used previously to inhibit stimulated release of NO (Handy *et al*., 1996) or acetylcholine-induced vasodilatation in rabbit isolated aorta (EC_{50} ~30 μM) (Moore *et al*., 1990) or to block basal release of NO from rat hearts (Pabla & Curtis, 1995). However, at least for L-NAME this argument might appear equivocal since L-NAME clearly is a prodrug of L-NOARG (Pfeiffer *et al*., 1996) and its rate of biotransformation may differ in different tissues leading to unequal inhibitory potencies. An alternative explanation would be that inhibition of NO synthase might not result in a measurable reduction of cyclic GMP in tissue homogenates if the steps leading to activation of guanylyl cyclase by NO are more complex than presently known, e.g. if storage forms of NO are present, as shown for endothelial cells (Davisson *et al*., 1996). This might explain why neither myocardial content of cyclic GMP nor its rate of release in coronary effluent (Figures 6 and 7) were altered by 1 μM L-NOARG while coronary flow was significantly reduced (Figure 1).

In several recent papers, the protective effect of NO synthase inhibition in the setting of cardiac ischaemia/reperfusion was attributed to an attenuated generation or action of oxygen-derived free radicals (Wang & Zweier, 1996; Yasmin *et al*., 1997). The theoretical basis for this interpretation is the reaction of NO with superoxide leading to the formation of peroxynitrite which is potentially injurious to tissues following its conversion to highly oxidant species (Beckman & Koppenol, 1996). Indeed, electron spin resonance studies in retrogradely perfused hearts of the rat (Zweier *et al*., 1989) and direct or indirect NO measurements (Depré & Hue, 1994; Maulik *et al*., 1995) suggest that NO release occurs during ischaemia, and peroxynitrite production during reperfusion (Yasmin *et al*., 1997). Whether free radicals were actually generated in the protocol used was not ascertained in our study; rather, we reduced the contribution of free-radical damage from our model by treating hearts and superoxide dismutase and catalase. Hearts were then perfused with these enzymes and L-NOARG to determine whether the NO synthase inhibitor could confer additional protection to hearts presumably relatively free of, at least some, oxygen-derived free radicals. The addition of L-NOARG to the perfusate during ischaemia gave no further protection to the heart and resulted in reperfusion functions of a similar magnitude. Therefore, L-NOARG might be acting by a similar mechanism as these enzymes although, admittedly, these data do not prove this interpretation because the individual interventions by themselves may have conferred the maximum possible protection (~80% recovery of LVDevP).

Finally, the present cyclic GMP data are relevant to the proposal of NO synthase inhibitor-mediated cardioprotection resulting from a reduced formation of peroxynitrite (Beckman & Koppenol, 1996). This free radical has generally been considered to be injurious to tissues, but was recently found to protect the ischaemic-reperfused rat heart by inhibiting neutrophil accumulation in cardiac tissue (Lefer *et al*., 1997). Irrespective of its actions, a reduced rate of formation of peroxynitrite from NO and superoxide is unlikely to account for the improved functional recovery observed in our experiments, because the increased formation of cyclic GMP in control hearts during ischaemia (Figure 6) and reperfusion (Figure 7) was not inhibited by L-NOARG at a low concentration.

Thus from the data presented here, we conclude that (a) both NO donors and NO synthase inhibitors in appropriate (but vastly different) concentrations attenuate ischaemic and reperfusion injury, (b) neither NO donors nor NO synthase inhibitors had any effect on tissue high energy phosphate levels, and (3) NO donors elevated myocardial cyclic GMP levels whereas low concentrations of NO synthase inhibitors did not reduce whole-heart or coronary effluent cyclic GMP levels. With respect to the apparent contradictory findings documented by other researchers and ourselves and aptly summarized in a recent review (Curtis & Pabla, 1997), the present data suggest that high dose NO donors act by modulating a cyclic GMP-dependent component, while NO synthase inhibitors may be cardioprotective via an additional mechanism independent of NO synthase activity.

The authors acknowledge the financial support of the Medical Research Council (MRC) of South Africa, Roche Products South Africa, the University of Cape Town, the Dr Heinrich-Jörg-Stiftung, Karl-Franzens-Universität Graz, Austria (F.B.), and the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (F.B.; grant 11040). In addition, the excellent technical assistance of Mr Gerald Wölkart is also acknowledged.

References

BARNES, C.S. & COKER, S.J. (1995). Failure of nitric oxide donors to alter arrhythmias induced by acute myocardial ischaemia or reperfusion in anaesthetized rats. *Br. J. Pharmacol.,* **114,** 349–356.

BECKMAN, J.S. & KOPPENOL, W.H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am. J. Physiol.,* **271,** C1424–C1437.

BRUNNER, F., STESSEL, H. & KUKOVETZ, W.R. (1995). Novel guanylyl cyclase inhibitor, ODQ reveals role of nitric oxide, but not of cyclic GMP in endothelin-1 secretion. *FEBS Lett.,* **376,** 262–266.

BUTT, E., EIGENTHALER, M. & GENIESER, H.-G. (1994). (Rp)-8-pCPT-cGMPS, a novel cGMP-dependent protein kinase inhibitor. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.),* **269,** 265–268.

CURTIS, M.J., MACLEOD, B.A., TABRIZCHI, R. & WALKER, J.A. (1986). An improved perfusion apparatus for small animal hearts. *J. Pharmacol. Meth.,* **15,** 87–94.

CURTIS, M.J. & PABLA, R. (1997). Nitric oxide supplementation or synthesis block - which is the better approach to treatment of heart disease? *Trends Pharmacol. Sci.,* **18,** 239–244.

DAVISSON, R.L., BATES, J.N., JOHNSON, A.K. & LEWIS, S.J. (1996). Use-dependent loss of acetylcholine- and bradykinin-mediated vasodilation after nitric oxide synthase inhibition: evidence for preformed stores of nitric oxide-containing factors in vascular endothelial cells. *Hypertension,* **28,** 354–360.

DEPRÉ, C. & HUE, L. (1994). Cyclic GMP in the perfused rat heart: effect of ischaemia, anoxia and nitric oxide synthase inhibitor. *FEBS Lett.,* **345,** 241–245.

DEPRÉ, C., VANOVERSCHELDE, J.-L., GOUDEMANT, J.-F., MOTTET, I. & HUE, L. (1995). Protection against ischemic injury by nonvasoactive concentrations of nitric oxide synthase inhibitors in the perfused rabbit heart. *Circulation,* **92,** 1911–1918.

EBIHARA, Y., HAIST, J.V. & KARMAZYN, M. (1996). Modulation of endothelin-1 effects on rat hearts and cardiomyocytes by nitric oxide and 8-bromo cyclic GMP. *J. Mol. Cell. Cardiol.,* **28,** 265–277.

EBIHARA, Y. & KARMAZYN, M. (1996). Inhibition of β- but not α_1-mediated adrenergic responses in isolated hearts and cardiomyocytes by nitric oxide and 8-bromo cyclic GMP. *Cardiovasc. Res.,* **32,** 622–629.

HANDY, R.L.C., HARB, H.L., WALLACE, P., GAFFEN, Z., WHITEHEAD, K.J. & MOORE, P.K. (1996). Inhibition of nitric oxide synthase by 1-(2-trifluoromethylphenyl)imidazole (TRIM) *in vitro*: antinociceptive and cardiovascular effects. *Br. J. Pharmacol.,* **119,** 423–431.

HARPER, J.F. & BROOKER, G. (1975). Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′O acetylation by the acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.,* **1,** 207–218.

JOHNSON, G., TSAO, P. & LEFER, A.M. (1990). Synergism between superoxide dismutase and sodium nitrite in cardioprotection following ischemia and reperfusion. *Am. Heart J.,* **119,** 530–537.

KANE, K.A., MORCILLO-SANCHEZ, E.J., PARRATT, J.R., RODGER, I.W. & SHAHID, M. (1985). The relationship between coronary artery occlusion-induced arrhythmias and myocardial cyclic nucleotide levels in the anaesthetized rat. *Br. J. Pharmacol.,* **84,** 139–145.

KELLY, R.A., BALLIGAND, J.-L. & SMITH, T.W. (1996). Nitric oxide and cardiac function. *Circ. Res.,* **79,** 363–380.

KING, L.M., BOUCHER, F. & OPIE, L.H. (1995). Coronary flow and glucose delivery as determinants of contracture in the ischemic myocardium. *J. Mol. Cell. Cardiol.,* **27,** 701–720.

KUKOVETZ, W.R., HOLZMANN, S., WURM, A. & PÖCH. G. (1979). Evidence for cyclic GMP-mediated relaxant effects of nitrocompounds in coronary smooth muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **310,** 129–138.

LAMPRECHT, W., STEIN, P., HEINZ, F. & WEISSER, H. (1974). Determination of creatine phosphate with creatine kinase, hexokinase, and glucose-6-phosphate dehydrogenase. In *Methods of Enzymatic Analysis*. ed. Bergmeyer, H.U. pp. 1777–1779. New York: Academic Press.

LAMPRECHT, W. & TRAUTSCHOLD, I. (1974). Determination of adenosine-5′-triphosphate with hexokinase and glucose-6-phosphate dehydrogenase. In *Methods of Enzymatic Analysis*. ed. Bergmeyer, H.U. pp. 2101–2110. New York: Academic Press.

LEFER, A.M. (1995). Attenuation of myocardial ischemia-reperfusion injury with nitric oxide replacement therapy. *Ann. Thorac. Surg.,* **60,** 847–851.

LEFER, A.M. & LEFER, D.J. (1996). The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. *Cardiovasc. Res.,* **32,** 743–751.

LEFER, D.J., NAKANISHI, K., JOHNSTON, W.E. & VINTEN-JOHANSEN, J. (1993). Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. *Circulation,* **88,** 2337–2350.

LEFER, D.J., SCALIA, R., CAMPBELL, B., NOSSULI, T., HAYWARD, R., SALAMON, M., GRAYSON, J. & LEFER, A.M. (1997). Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia-reperfusion injury in rats. *J. Clin. Invest.,* **99,** 684–691.

LINZ, W., WIEMER, G. & SCHÖLKENS, B.A. (1992). ACE-inhibition induces NO-formation in cultured bovine endothelial cells and protects isolated ischemic rat hearts. *J. Mol. Cell. Cardiol.,* **24,** 909–919.

MATHEIS, G., SHERMAN, M.P., BUCKBERG, G.D., HAYBRON, D.M., YOUNG, H.H. & IGNARRO, L.J. (1992). Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am. J. Physiol.,* **262,** H616–H620.

MAULIK, N., ENGELMAN, D.T., WATANABE, M., ENGELMAN, R.M., MAULIK, G., CORDIS, G.A. & DAS, D.K. (1995). Nitric oxide signaling in ischemic heart. *Cardiovasc. Res.,* **30,** 593–601.

MÉRY, P.-F., PAVOINE, C., BELHASSEN, L., PECKER, F. & FISCHMEISTER, R. (1993). Nitric oxide regulated Ca^{2+} current: involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J. Biol. Chem.,* **268,** 26286–26295.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.,* **99,** 408–412.

OPIE, L.H. (1989). Reperfusion injury and its pharmacological modification. *Circulation,* **80,** 1049–1062.

PABLA, R., BLAND-WARD, P., MOORE, P.K. & CURTIS, M.J. (1995). An endogenous protectant effect of cardiac cyclic GMP against reperfusion-induced ventricular fibrillation in the rat heart. *Br. J. Pharmacol.,* **116,** 2923–2930.

PABLA, R., BUDA, A.J., FLYNN, D.M., BLESSÉ, S.A., SHIN, A.M., CURTIS, M.J. & LEFER, D.J. (1996). Nitric oxide attenuates neutrophil-mediated myocardial contractile dysfunction after ischemia and reperfusion. *Circ. Res.,* **78,** 65–72.

PABLA, R. & CURTIS, M.J. (1995). Effects of NO modulation on cardiac arrhythmias in the rat isolated heart. *Circ. Res.,* **77,** 984–992.

PABLA, R. & CURTIS, M.J. (1996). Effect of endogenous nitric oxide on cardiac systolic and diastolic function during ischemia and reperfusion in the rat isolated perfused heart. *J. Mol. Cell. Cardiol.,* **28,** 2111–2121.

PFEIFFER, S., LEOPOLD, E., SCHMIDT, K., BRUNNER, F. & MAYER, B. (1996). Inhibition of nitric oxide synthase by N^G-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, N^G-nitro-L-arginine. *Br. J. Pharmacol.,* **118,** 1433–1440.

RICHARD, V., KAEFFER, N., TRON, C. & THUILLEZ, C. (1994). Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation,* **89,** 1254–1261.

SAIDA, K., UMEDA, M. & ITAKURA, A. (1994). Improvement of ischemic myocardial dysfunction by nisoldipine. *Cardiovasc. Drugs Ther.,* **8,** 365–370.

SATO, H., ZHAO, Z.-Q., MCGEE, D.S., WILLIAMS, M.W., HAMMON, J.W. & VINTEN-JOHANSEN, J. (1995). Supplemental L-arginine during cardioplegic arrest and reperfusion avoids regional postischemic injury. *J. Thorac. Cardiovasc. Surg.,* **110,** 302–314.

SCHULZ, R. & WAMBOLT, R. (1995). Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. *Cardiovasc. Res.,* **30,** 432–439.

SHAH, A.M., SPURGEON, H.A., SOLLOTT, S.J., TALO, A. & LAKATTA, E.G. (1994). 8-Bromo-cGMP reduces the myofilament response to Ca^{2+} in intact cardiac myocytes. *Circ. Res.,* **74,** 970–978.

SIEGFRIED, M.R., ERHARDT, J., RIDER, T., MA, X.-L. & LEFER, A.M. (1992). Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischaemia-reperfusion. *J. Pharmacol. Exp. Ther.,* **260,** 668–675.

VANOVERSCHELDE, J.-L.J., JANIER, M.F. & BERGMANN, S.R. (1994). The relative importance of myocardial energy metabolism compared with ischemic contracture in the determination of ischemic injury in isolated perfused rabbit hearts. *Circ. Res.,* **74,** 817–828.

WAHLER, G.M. & DOLLINGER, S.J. (1995). Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am. J. Physiol.,* **268,** C45–C54.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.,* **39,** 163–196.

WANG, P. & ZWEIER, J.L. (1996). Measurement of nitric oxide and peroxynitrite generation in the postischemic heart: evidence for peroxynitrite-mediated reperfusion injury. *J. Biol. Chem.,* **271,** 29223–29230.

WEYRICH, A.S., MA, X.-L. & LEFER, A.M., (1992). The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation,* **86,** 279–288.

WODITSCH, I. & SCHRÖR, K. (1992). Prostacyclin rather than endogenous nitric oxide is a tissue protective factor in myocardial ischemia. *Am. J. Physiol.,* **263,** H1390–H1396.

YANG, B.C., CHEN, L.Y., SALDEEN, T.G.P. & MEHTA, J.L. (1997). Reperfusion injury in the endotoxin-treated rat heart: reevaluation of the role of nitric oxide. *Br. J. Pharmacol.,* **120,** 305–311.

YANG, B.C., VIRMANI, R., NICHOLS, W.W. & MEHTA, J.L. (1993). Platelets protect against myocardial dysfunction and injury induced by ischemia and reperfusion in isolated rat hearts. *Circ. Res.,* **72,** 1181–1190.

YASMIN, W., STRYNADKA, K.D. & SCHULZ, R. (1997). Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc. Res.,* **33,** 422–432.

ZWEIER, J.L., KUPPUSAMY, P., WILLIAMS, R., RAYBURN, B.K., SMITH, D., WEISFELDT, M.L. & FLAHERTY, J.T. (1989). Measurement and characterization of postischemic free radical generation in the isolated perfused heart. *J. Biol. Chem.,* **264,** 18890–18895.

(Received July 3, 1997
Revised October, 28, 1997
Accepted November 28, 1997)