British Journal of Pharmacology (1999) 128, 891–902
http://www.stockton-press.co.uk/bjp

Deficiency in myocardial NO biosignalling after cardioplegic arrest: mechanisms and contribution to post-storage mechanical dysfunction

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1 In order to understand mechanisms that limit the safe ischaemic time of donor hearts, this study evaluated NO/cyclic GMP biosignalling in the recovery of function after cardioplegia and hypothermic storage.

2 Hearts removed from anaesthetized rats were either perfused in working mode (Fresh) or arrested (St. Thomas' II cardioplegia) and stored at 3°C for 8 h (CPL) prior to working mode perfusion. LV work and indices of the production of NO (Ca^{2+}-dependent and Ca^{2+}-independent NOS), cyclic GMP (soluble guanylyl cyclase (sGC) and GTP) and superoxide (xanthine oxidase (XO) and xanthine dehydrogenase (XDH)) were measured.

3 Relative to Fresh hearts, CPL hearts were deficient in cyclic GMP and had poor function. Correction of cyclic GMP deficiency (SNP, 200 μM) improved LV work and LV compliance. SNP effects were prevented by inhibition of sGC (ODQ, 3 μM), and potentiated by inhibition of cyclic GMP-dependent phosphodiesterase (zaprinast, 20 μM). SNP (200 μM) had no effect on function of Fresh hearts.

4 NOS activities (pH = 7.2) were similar in CPL and Fresh hearts, but at end-ischaemic pH (6.3), Ca^{2+}-dependent NOS activity was reduced. The sensitivity of sGC to SNP was greater, and activities of XO and XDH were higher, in CPL than in Fresh hearts.

5 The deficiency in NO biosignalling in CPL hearts may arise due to acidosis-induced inhibition of NOS activity, reduced availability of GTP and/or enhanced inactivation of NO by superoxide. These findings provide rationales for novel strategies to prevent the deficiency in NO biosignalling and so improve the function of the transplanted heart.

Keywords: Cardioplegia; myocardial function; working rat hearts; nitric oxide; cyclic GMP

Abbreviations: AF, aortic flow; ANOVA, analysis of variance; ATP, adenosine triphosphate; cAMP, cyclic adenosine 3′5′-monophosphate; CF, coronary flow; cGMP, cyclic guanosine 3′5′-monophosphate; CO, cardiac output; CPL, cardioplegia; CVC, coronary vascular conductance; DTT, dithiotreitol; DMSO, dimethylsulphoxide; EC_{50}, concentration eliciting 50% of maximum response; EGTA, ethylene glycol-bis[β-aminoethyl ether]-N,N,N′N′-tetraacetic acid; EIA, enzyme immunoassay; eNOS, Ca^{2+}-dependent nitric oxide synthase; GTP, guanosine triphosphate; HPLC, high pressure liquid chromatograph; iNOS, Ca^{2+}-independent nitric oxide synthase; K_m, concentration of substrate required for 50% maximal velocity; L-NMMA, N-monomethyl-L-arginine; LV, left ventricular; LVEDP, left ventricular end diastolic pressure; Na_2EDTA, ethylenediamineteytraacetate, disodium salt; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PCA, perchloric acid; PCr, creatine phosphate; PMSF, phenylmethanesulphonyl fluoride, sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; XO, xanthine oxidase; XDH, xanthine dehydrogenase

Introduction

Cardiac transplantation is a life-saving surgical procedure for patients with end-stage heart disease, but graft availability is limited and procurement of viable donor organs is contingent upon implantation within the 'safe' ischaemic time of less than 4–6 h. Furthermore, despite improvements in the composition of cardioplegic solutions, inadequate graft preservation during ischaemia continues to have a significant impact on early morbidity after cardiac transplantation (Hauptman *et al*., 1994). Pharmacological interventions to enhance mechanical function of the donor heart can be delivered at the time of organ extraction, during storage and/or during early reperfusion. However, development of cardioprotective drugs to extend the safe ischaemic time of the donor heart is limited by a poor understanding of the underlying mechanisms that contribute to impaired recovery of mechanical function of donor hearts following prolonged hypothermic storage.

Recent evidence suggests an association between poor recovery of post-ischaemic myocardial mechanical function and diminished bioavailability of nitric oxide (NO) (Ma *et al*., 1993; Pinsky *et al*., 1994; Nakanishi *et al*., 1995; Lefer, 1995; Lefer & Lefer, 1996). Impaired bioavailability of endothelial NO, that results in coronary vasomotor dysfunction, has been implicated in injury occurring during reperfusion even when hearts are preserved under the 'best available' conditions employing cardioplegic solutions (Pearl *et al*., 1994). Supporting the importance of NO bioavailability, administration of exogenous NO donors is cardioprotective against short-term ischaemia (Nakanishi *et al*., 1995; Zhao *et al*., 1996), enhances recovery of mechanical function following cardioplegic arrest

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and prolonged (8 h) hypothermic storage (Ali *et al*., 1998), and improves graft survival (Pinsky *et al*., 1994). Strategies that enhance endogenous NO production are also protective (Williams *et al*., 1995). For example, monophosphoryl lipid A (MLA) (Zhao *et al*., 1997) or lipopolysaccharide (Yang *et al*., 1997) administered 12–24 h prior to ischaemia, enhances myocardial NO synthase (NOS) activity, reduces myocardial infarct size and improves recovery of post-ischaemic mechanical function. Whether cardioprotection by both exogenous and endogenous NO results from a correction of a deficiency in NO production and/or by stimulating myocardial or coronary NO/cyclic GMP transduction pathways is unknown.

Mechanisms that have been considered for the beneficial actions of NO on cardiac function include enhanced myocardial perfusion due to coronary vasodilatation and/or inhibition of endothelial-neutrophil interactions (Hansen, 1995; Lefer, 1997). Considerable benefit is observed in *in vivo* preparations where NO is believed to inhibit neutrophil-induced damage in the coronary circulation and so preserve endothelial function and O_2 and energy substrate delivery (Lefer & Lefer, 1996). However, recently we (Ali *et al*., 1998) and others have shown that the cardioprotective effects of the NO-donors, sodium nitroprusside (SNP) (Grocott-Mason *et al*., 1994) or nitroglycerin (Ferdinandy *et al*., 1995), are independent of changes in myocardial perfusion. Moreover, we have also shown that the specific soluble sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Moro *et al*., 1996), antagonizes SNP-induced protection in hearts perfused with crystalloid perfusate that was devoid of blood-borne cellular elements. These data provide indirect evidence that a component of the cardioprotective action of NO is elicited directly on cardiac muscle and arises as a consequence of the activation of soluble guanylyl cyclase (sGC).

In contrast, adverse effects of NO on the recovery of post-ischaemic mechanical function have been reported based on studies in which inhibition of NOS activity and attenuated NO production improve post-ischaemic function (Morita *et al*., 1995; Schulz & Wambolt, 1995). Recent evidence indicates that such protection is due to impaired production of peroxynitrite, a toxic product arising from the chemical interaction of NO with superoxide anion (Yasmin *et al*., 1997). Consequently, while NO-mediated biosignalling depends upon NO availability (balance between NO production and inactivation) as well as the sensitivity of sGC, their integrity in hearts subjected to prolonged hypothermic storage and their contribution to post-storage mechanical dysfunction have not been defined.

This study first investigated the relationship between drug-induced perturbation of myocardial cyclic GMP content and recovery of post-ischaemic mechanical function in order to determine if a deficiency in NO biosignalling contributed to the impaired function of hearts subjected to cardioplegic arrest and prolonged hypothermic storage. Thereafter, the activities of enzyme systems responsible for myocardial NO generation (Ca^{2+}-dependent and Ca^{2+}-independent NOS), superoxide anion production (xanthine oxidase (XO) and xanthine dehydrogenase (XDH)) as well as cyclic GMP generation (sGC) were measured in fresh hearts and hearts subjected to cardioplegic arrest and prolonged hypothermic storage. Enzyme assays were performed at normal pH as well as at the pH associated with end-storage conditions (determined by NMR spectroscopy). With this approach, it was possible to assess whether changes in NO production, NO inactivation and/or sGC sensitivity contributed to the alterations in myocardial NO biosignalling.

Methods

Isolated perfused rat hearts

Male Sprague-Dawley rats aged 7–8 weeks and weighing 300–350 g were used. All animals were fed *ad libitum* and received care according to the Canadian Council on Animal Care and the Health Sciences Animal Policy and Welfare Committee, University of Alberta, Canada. Hearts were excised following euthanasia with sodium pentobarbitone (80 mg kg^{-1}, i.p.). The aortae were cannulated and all hearts were perfused, without pacing, in the Langendorff (L) mode at a constant perfusion pressure of 60 mmHg for 10 min as described previously (Finegan *et al*., 1996; Ali *et al*., 1998). The Langendorff perfusate consisted of a Krebs-Henseleit solution (37°C, pH 7.4 gassed with a 95% O_2/5% CO_2 mixture) that contained 11 mM glucose and 2.5 mM Ca^{2+} (Figure 1). After an initial 10-min Langendorff perfusion, hearts were assigned to either a Cardiopleged group (CPL) or a Fresh group and perfused as follows:

CPL hearts After the initial 10-min Langendorff mode perfusion, hearts were arrested with ice-cold St. Thomas' II cardioplegic solution (mM): NaCl 110, $NaHCO_3$ 10, KCl 16, $MgCl_2.6H_2O$ 16 and $CaCl_2$ 1.2, pH 7.8, ungassed, which was administered at a constant perfusion pressure of 60 mmHg. Following delivery of 25 ml of cardioplegic solution, hearts were removed from the perfusion rig, immersion stored in St. Thomas' II solution at 3 ± 1°C for 8 h (Storage Phase) and then re-warmed, without pacing, during a 10-min Langendorff mode reperfusion with Krebs-Henseleit solution at 37°C (Figure 1). Hearts were then either rapidly frozen with Wollenberger clamps cooled to the temperature of liquid nitrogen for biochemical measurements or they were subjected to aerobic working mode reperfusion for 60 min. Reperfusion in the working mode was performed at constant left atrial preload (11.5 mmHg) and aortic afterload (80 mmHg). During working mode hearts were paced at 300 beats min^{-1} (Grass SD9 Stimulator). The reperfusion fluid (recirculating volume of 100 ml) consisted of a modified Krebs-Henseleit solution at 37°C that contained 2.5 mM Ca^{2+}, 11 mM glucose, 100 μu ml^{-1} insulin and 1.2 mM palmitate pre-bound to 3% bovine serum albumin (BSA, fraction V). CPL hearts were either untreated, or exposed to SNP for specific phases of the perfusion protocol (Figure 1) in the absence or presence of an inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 3 μM) or cyclic GMP phosphodiesterase, zaprinast (20 μM).

Fresh hearts After the initial 10-min Langendorff perfusion, hearts were either rapidly frozen for biochemical measurements or were perfused in the aerobic working mode for 60 min at constant left atrial preload, aortic afterload and rate as described above (Figure 1). The direct effects of SNP on mechanical function of hearts perfused under normal aerobic conditions were also assessed.

Assessment of LV mechanical function

During working mode reperfusion of CPL hearts and throughout working mode perfusion of Fresh hearts, systolic and diastolic aortic pressures (mmHg) were measured using a Gould P21 pressure transducer attached to the aortic outflow line. Cardiac output (CO, ml min^{-1}) and aortic flow (AF, ml min^{-1}) were measured using ultrasonic flow probes (Transonic T206) placed in the left atrial inflow line and aortic outflow

Table 1 Assessment of mechanical function of hearts at the end of 60 min of working mode perfusion

Treatment groups (n)	*LV work* (Joules)	*Cardiac output* (ml min^{-1})	*Aortic flow* (ml min^{-1})	*Coronary flow* (ml min^{-1})	*Coronary vascular conductance* (ml min^{-1} $mmHg^{-1}$)
Fresh (6)	0.87±0.08	60.3±4.3	40.8±4.8	19.5±4.4	0.235±0.055
CPL (8)	0.21±0.07	18.6±5.5	7.6±3.6	11.0±2.3	0.162±0.020
Effect of ODQ treatment during Langendorff reperfusion					
SNP 200 μM (8)	0.56±0.05	44.5±3.5*	22.0±3.3*	22.5±0.9*	0.282±0.015*
ODQ 3 μM (8)	0.36±0.11	27.8±7.7	15.5±4.6	12.3±3.2	0.178±0.024*
SNP+ODQ (8)	0.34±0.06	27.8±4.0$	11.5±2.1$	16.3±2.3$	0.214±0.017$
Effect of zaprinast treatment when present during all phases of perfusion					
SNP 50 μM (8)	0.37±0.08	29.9±5.9	12.0±4.8	17.9±2.0	0.227±0.020
ZAP 20 μM (4)	0.22±0.06	20.3±4.6	3.8±2.3	16.5±3.2	0.217±0.046
SNP+ZAP (8)	0.67±0.07*$	45.0±3.4*$	23.1±3.1*	21.9±0.7*	0.243±0.009*
SNP 200 μM (8)	0.72±0.08*	52.9±4.9*	27.9±3.2*	25.0±3.2*	0.301±0.039*

Values are means±s.e.mean. Significant differences between untreated and treated CPL hearts are indicated by *, whereas differences between groups treated with SNP and those perfused with SNP in combination with ODQ (3 μM) or zaprinast (ZAP, 20 μM) are indicated by $.

Table 2 Effects of SNP on mechanical function of fresh hearts during aerobic working mode perfusion

Treatment groups (n)	*LV work* (Joules)	*Cardiac output* (ml min^{-1})	*Aortic flow* (ml min^{-1})	*Coronary flow* (ml min^{-1})	*Coronary vascular conductance* (ml min^{-1} $mmHg^{-1}$)
Control (4)	1.10±0.09	66.5±3.7	47.5±4.7	19.0±2.1	0.187±0.104
SNP 200 μM (8)	1.10±0.04	67.4±1.6	45.9±2.5	21.5±1.3	0.213±0.012

Values are means±s.e.mean of functional parameters after a total of 60 min of wroking mode perfusion. SNP, when present, was added to the perfusate at a final concentration of 200 μM after 40 min of baseline perfusion. There were no significant differences between Control and SNP-treated groups.

line, respectively. Coronary flow (CF, ml min^{-1}) was calculated from the difference between CO and AF. Left ventricular minute work (LV work, Joules), calculated as CO × LV developed pressure, served as a continuous index of mechanical function. Coronary vascular conductance (CVC, ml min^{-1} $mmHg^{-1}$) was calculated as the ratio of CF and mean aortic pressure.

Assessment of LV compliance

Changes in LV pressure occurring in response to graded increases in LV volume were used to assess LV compliance of CPL hearts (Larson *et al*., 1998). Additional groups of CPL hearts, that were either untreated or treated with SNP (200 μM) during storage and Langendorff mode reperfusion, were used. LV end diastolic pressure (LVEDP) and LV systolic pressure were measured between 5 and 10 min of Langendorff reperfusion with a pressure transducer that was connected to a water-filled balloon (Radinoti #6, Harvard Apparatus Inc.) that had been passed through the left atrium and positioned in the LV. A syringe was attached to the balloon and was used to increase LV volume cumulatively in 10 μl increments every 30 s. The associated changes in LVEDP (mmHg) in response to these graded increases in LV volume (μl) were recorded. Hearts were paced at 300 beats min^{-1} during this procedure.

Assessment of intracellular pH and ATP by NMR spectroscopy

Measurements of intracellular pH and ATP during Langendorff mode reperfusion of additional groups of Fresh and untreated CPL hearts were performed by NMR spectroscopy. Experiments were performed on a Bruker AM-360 spectrometer in conjunction with a wide bore 8.7 Tesla superconducting magnet (Oxford Magnets, Oxford, U.K.) and equipped with a broadband probe. Field homogeneity was adjusted by shimming on the Na^+ signal from the sample yielding line widths of 10–15 Hz. [^{31}P]-NMR spectra were acquired at 145.8 MHz with a 60° pulse. Seventy-two free induction decays were summed to give a time resolution of 2.5 min. The sweep width was 10 kHz and 4096 data points were collected. Spectra were processed by Fourier transformation following exponential multiplication (line broadening of 20 Hz). A capillary containing phenyl phosphonic acid was placed next to the heart and acted as an internal reference. The content of ATP was determined from the height of the β-ATP peak at −16 p.p.m. (with PCr assigned at 0 p.p.m.). Intracellular pH was determined from the chemical shift of P_i relative to phosphocreatine (Moon & Richards, 1973) with a calibration curve obtained by titrating P_i in a solution mimicking the intracellular milieu (Stewart *et al*., 1994). The calibration curve fitted the Henderson-Hasselbach equation with the following parameters: $\Delta A = 3.30$ p.p.m., $\Delta B = 5.75$ p.p.m. and $pK_2 = 6.85$.

Hearts were cannulated and subjected to Langendorff mode perfusion, placed in a 20 mm NMR tube and immediately inserted into the magnet. Following shimming of the magnet and tuning of the probe, two baseline ^{31}P spectra were acquired to provide aerobic baseline values for intracellular pH and ATP in Fresh hearts.

The hearts were then removed from the magnet and then arrested (as described above) with ice-cold St Thomas' solution and immersion stored in St Thomas' solution for 8 h at 3°C. At the end of the storage period, hearts were remounted on the perfusion cannulae, placed within the NMR tube, immersed in ice-cold St Thomas' solution and re-inserted into the bore of the magnet. Shimming was checked by acquiring a sodium spectrum *via* the dual channel probe from four FIDs and ensuring that the line width was not significantly altered from the pre-storage condition. The tuning of the phosphorus

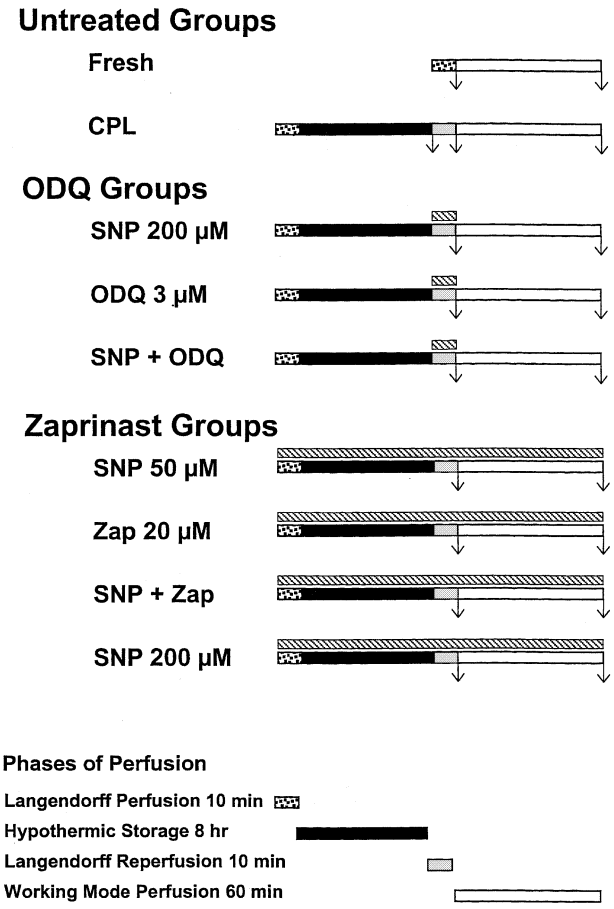

Figure 1 Diagram of the perfusion protocols used to assess LV mechanical function in the absence and presence of drug treatments. Fresh hearts were rapidly isolated and immediately subjected to 10-min Langendorff perfusion. These hearts were then either rapidly frozen (indicated by ↓) for biochemical measurements or were perfused in aerobic working mode for 60 min for the measurement of mechanical function. CPL hearts were isolated in a similar manner, perfused for 10 min in Langendorff mode, arrested with 25 ml ice-cold St. Thomas' II cardioplegic solution and stored in St. Thomas' II solution at 3 ± 1°C for 8 h. CPL hearts were re-warmed to 37°C during 10-min Langendorff reperfusion when measurements of pH and LV compliance were made. After this phase, hearts were either rapidly frozen for biochemical measurements or were subjected to aerobic working mode reperfusion for 60 min to assess post-storage LV mechanical function. CPL hearts in the ODQ groups were perfused in a similar manner except that hearts were exposed to SNP, ODQ or SNP in combination with ODQ during only the Langendorff reperfusion phase as indicated by the hatched bar. CPL hearts in the Zaprinast groups were also perfused in a similar manner except that hearts were exposed to SNP, zaprinast or SNP in combination with zaprinast throughout all phases of the perfusion protocol as indicated by the hatched bar.

channel was then checked by attaching to a frequency sweeper box (Morris Instruments Inc.) to ensure that tuning and matching had not changed from pre-storage conditions. Two ^{31}P NMR spectra were then acquired to assess the intracellular pH and ATP at the end of the storage period. Hearts were then reperfused with Krebs-Henseleit solution for 10 min in Langendorff reperfusion mode while acquiring ^{31}P NMR spectra in order to monitor the recovery of intracellular pH and ATP.

Assay of cyclic nucleotide contents

Frozen ventricles were powdered with a mortar and pestle maintained at the temperature of liquid nitrogen. The myocardial contents of cyclic GMP and cyclic AMP in Fresh and CPL hearts were determined by commercially available enzyme immunoassay kits (Cayman Chemical, Ann Arbour, MI, U.S.A.). Frozen heart powder (200–250 mg) was homogenized with 500 μl HEPES PCA (2.2%) and 25 μl Na_2EDTA (5 mM). Samples (250 μl) of supernatant were neutralized with 40 μl K_3PO_4 to pH of 7 to 8 and extracts were centrifuged ($10{,}000 \times g$ for 2 min at 4°C). Samples (50 μl) of the resulting supernatant were used for the determination of cyclic GMP and cyclic AMP. The protein in the remaining pellet was hydrolyzed with NaOH (2 M), neutralized with HCl to pH 7 and the protein content was measured by the microplate BCA method. The contents of cyclic GMP and cyclic AMP are expressed as pmol mg protein^{-1} (Moro *et al.*, 1996).

Assay of high-energy nucleotides

Samples of frozen heart powder (100 mg) were homogenized in 1 ml of ice-cold PCA (6%)/EGTA (0.5 mM). Homogenates were centrifuged ($10{,}000 \times g$ for 10 min) and the resulting supernatants were neutralized with 50 μl K_2CO_3 (5 M) and re-centrifuged ($10{,}000 \times g$ for 10 min). The supernatant was then analysed for high-energy nucleotide content by reverse-phase HPLC (Ally & Park, 1992). A small sample of frozen powdered tissue (100 mg) was weighed before and after 24 h of drying in a warm oven. The difference in weight was used to calculate a dry weight to wet weight ratio for each heart; this enabled high-energy nucleotide contents to be expressed as μmol g dry wt^{-1}.

Assay of NOS activities

Samples of frozen heart powder (150–200 mg) were homogenized (1 : 4 w v^{-1}) in HEPES buffer (10 mM) containing sucrose (320 mM), DTT (1 mM), leupeptin (10 μg ml^{-1}), soybean trypsin inhibitor (10 μg ml^{-1}) and aprotinin (2 μg ml^{-1}) using an ultrasonic processor and centrifuged at $10{,}000 \times g$ for 15 min at 4°C. Crude homogenate (20 μl) was incubated for 25 min at 37°C in assay medium (Schulz *et al.*, 1992) under three conditions as follows: assay cocktail alone, assay cocktail containing EGTA (1 mM), or assay cocktail containing EGTA (1 mM) and L-NMMA (1 mM). In this way, the activity of the two isoforms, eNOS (Ca^{2+}-dependent) and iNOS (Ca^{2+}-independent) were measured from the production of L-[^{14}C]citrulline from L-[^{14}C]arginine (Schulz *et al.*, 1992). Assays were performed at pH = 7.2 as well as at pH = 6.3 to mimic fresh and post-storage conditions. Separation of L-[^{14}C]citrulline from L-[^{14}C]arginine was achieved by incubating assay mixtures with cation exchange resin (AG 50W-X8, Bio-Rad Laboratories, CA, U.S.A.) to remove unreacted L-[^{14}C]arginine. Following centrifugation ($10{,}000 \times g$ for 2 min at room temperature), the L-[^{14}C]citrulline remaining in the supernatant was determined by liquid scintillation counting. Protein contents of the homogenates (20 μl) were measured by the BCA method and all NOS activities were expressed as pmol L-citrulline formed min^{-1} mg protein^{-1}.

Assay of soluble guanylyl cyclase (sGC) sensitivity

The sensitivity of sGC to NO-induced stimulation was measured in cytosolic extracts of frozen samples of Fresh and untreated CPL hearts by their ability to manufacture cyclic GMP in response to activation by graded concentrations of SNP using a modification of a previously described method for cardiac myocytes (Davis *et al.*, 1997). Frozen heart powder

(50–100 mg) was homogenized (1 : 10 w v^{-1}) in HEPES buffer (50 mM) containing DTT (1 mM) and PMSF (0.1 mM) and centrifuged (100,000 × *g* for 35 min at 4°C). Aliquots of the resulting supernatant (30 μl) containing 1.5–2.0 mg ml^{-1} of protein were incubated with an assay mixture containing GTP (0.2 mM) for 10 min at 37°C in the presence of graded concentrations of SNP (0–1000 μM), either at pH = 7.2 or pH = 6.3. The reaction was stopped by the addition of 50 μl PCA (2.2%) and 12.5 μl Na_2EDTA (5 mM). Following neutralization with K_3PO_4 and centrifugation (10,000 × *g* at 4°C), the cyclic GMP produced was assayed by the EIA method described above. Protein contents of the supernatants were assayed by the BCA method. The activity of sGC is expressed as pmol cyclic GMP min^{-1} mg $protein^{-1}$.

Assay of xanthine oxidoreductase (XOR) activity

Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) activities in untreated CPL and Fresh hearts were assayed by measuring the conversion of pterin to the fluorescent product, isoxanthopterin, using a spectrofluorometric assay (Beckman *et al*., 1989). Samples of frozen heart tissue (150 mg) were homogenized (1 : 4 w v^{-1}) with ice-cold HEPES buffer (10 mM) containing sucrose (320 mM), DTT (1 mM), leupeptin (10 μg ml^{-1}), soybean trypsin inhibitor (10 μg ml^{-1}), aprotinin (2 μg ml^{-1}), EDTA (0.1 mM) and PMSF (0.1 mM) and centrifuged at 100,000 × *g* for 35 min at 4°C. Aliquots (100 μl) of the resulting supernatant were gently mixed with KH_2PO_4 buffer (50 mM, pH = 7.2) in a spectrofluorimeter quartz cuvette. After 2-min of baseline observation, pterin (final concentration 10 μM) was added and the gradual linear increase in fluorescence obtained during the subsequent 2 min was used to calculate XO activity of the sample. Methylene blue (final concentration 10 μM) was then added and the associated more rapid increase in fluorescence was used to calculate the combined activities of XO and XDH (total XOR activity). The reaction was stopped by the addition of allopurinol (50 μM). To calibrate the fluorescence measurements, the activity of a standard concentration of isoxanthopterin (0.1 μM) was measured. XDH activity was calculated as the difference between XOR and XO activities and all activities are expressed as pmol isoxanthopterin min^{-1} mg $protein^{-1}$. Measurement of the activities of XO and XDH at pH = 6.3 was not attempted in light of previously reported technical difficulties (Beckman *et al*., 1989).

Drugs and reagents

Sodium nitroprusside (SNP, Hoffmann-La Roche), which was selected as a robust NO-donor that can spontaneously release NO without the need for enzymatic conversion, was dissolved in Krebs-Henseleit solution, St. Thomas' II cardioplegic solution or assay solutions as required by the experimental protocol. The perfusion apparatus, storage bottles and assay containers were covered with aluminum foil to protect SNP from light-induced decomposition. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was purchased from Tocris Cookson Inc., U.S.A., and was dissolved in 100 μl DMSO and diluted in Krebs-Henseleit solution to achieve a final concentration of 3 μM (final DMSO of 0.005%). Zaprinast, which was purchased from Research Biochemicals International, was dissolved in 1 M NaOH and added at a 10,000 fold dilution to Krebs-Henseleit or St. Thomas' II cardioplegia as required to achieve a final concentration of 20 μM (Wood & Owen, 1989). L-arginine and L-[U-^{14}C]arginine (50 μCi ml^{-1}) were purchased from Fluka Chemika-BioChemika, U.S.A. and Amersham Life Science, respectively. The remainder of the chemicals was purchased from Sigma Chemical Co, Canada. All perfusate solutions were filtered before (Whatman GF/C) and during (in-line glass filter) heart perfusion.

Statistical analysis

All data are expressed as mean ± s.e.mean. The significance of differences among groups was estimated by ANOVA. If significant, selected data sets were compared by Bonferroni Multiple Comparisons test following confirmation by Bartlett's test that variances were homogeneous. Differences were considered significant when $P < 0.05$.

Results

Myocardial content of cyclic nucleotides

Comparison of the cyclic GMP content of Fresh hearts ($n = 6$) after Langendorff mode perfusion with untreated CPL hearts ($n = 6$) frozen after 8 h hypothermic storage and 10 min of Langendorff mode reperfusion indicated that CPL hearts were deficient in cyclic GMP content and had only 49% of the content of Fresh hearts (Figure 2A). The presence of SNP (200 μM, $n = 4$) during only the 10-min period of Langendorff mode reperfusion elevated the content of cyclic GMP by 320% relative to untreated CPL hearts. The soluble guanylyl cyclase inhibitor, ODQ (3 μM), had no effect *per se* on cyclic GMP content, but when present during only the 10-min period of Langendorff mode reperfusion ($n = 4$), it completely inhibited the SNP-mediated elevation of cyclic GMP content (Figure 2A).

When present throughout all phases of perfusion, SNP (50 and 200 μM, $n = 4$ per group) elicited a concentration-dependent increase in cyclic GMP content (Figure 2B). Although the cyclic GMP phosphodiesterase inhibitor, zaprinast (20 μM) when present throughout all phases of perfusion did not alter cyclic GMP content, in combination with SNP (50 μM, $n = 5$), it significantly enhanced the stimulant effect of this sub-maximal concentration of SNP by 232% to a level equivalent to the action of SNP (200 μM, $n = 4$) alone (Figure 2B).

The content of cyclic AMP (pmol mg $protein^{-1}$) was similar in Fresh (3.7 ± 0.2, $n = 6$) and untreated CPL hearts (3.8 ± 0.1, $n = 6$). Moreover, myocardial cyclic AMP contents were not influenced by SNP (50 or 200 μM, $n = 4$ per group), either in the absence or presence of ODQ or zaprinast (data not shown).

Recovery of post-storage LV mechanical function

In hearts subjected to cardioplegic arrest, hypothermic storage at 3°C for 8 h, Langendorff mode reperfusion for 10 min and then 60 min working mode reperfusion, LV work recovered to only 24% ($n = 8$) of the LV work level measured in Fresh hearts ($n = 6$) (Ali *et al*., 1998). The addition of SNP (200 μM, $n = 8$) only during the 10-min period of Langendorff reperfusion increased the subsequent recovery of LV work to 65% of the LV work level measured in Fresh hearts (Figure 2C). ODQ (3 μM) *per se* had no effect on the extent of recovery of mechanical function, but it completely antagonized the beneficial effect of SNP ($n = 8$) (Figure 2C, Table 1).

As reported previously (Ali *et al*., 1998), when CPL hearts were treated with SNP (50 and 200 μM, $n = 8$ per group) during extraction, storage, Langendorff and mode working reperfusion, a significant increase in the recovery of LV work to 83%

of the LV work level of Fresh hearts was obtained only with the higher concentration. With the lower concentration of SNP (50 μM) there was a tendency for improved recovery of function, but the difference was not significant (Figure 2D). Recovery of LV work was not affected by zaprinast *per se*, but the effect of the previously ineffective concentration of SNP was potentiated by zaprinast so that the recovery of LV work in the combined presence of SNP (50 μM) and zaprinast (20 μM) increased significantly to 77% ($n=8$) of that measured in Fresh hearts (Figure 2D, Table 1).

Intracellular pH and ATP by NMR spectroscopy

Intracellular pH was assessed by NMR spectroscopy prior to storage, at the end of the 8 h period of hypothermic storage and during the 10-min period of Langendorff mode reperfusion in untreated CPL hearts. Pre-storage aerobic baseline intracellular pH was 7.16 ± 0.02 ($n=4$). After cardioplegic arrest and 8 h of hypothermic storage, intracellular pH was reduced to 6.35 ± 0.06 ($n=7$). During Langendorff mode reperfusion, intracellular pH recovered rapidly (6.92 ± 0.09, $n=4$, at 2.5 min; 7.17 ± 0.04, $n=4$ at 5 min) and returned to normal pre-storage values by 7.5 min of reperfusion (7.20 ± 0.02, $n=4$). Similarly, ATP contents as determined by NMR spectroscopy (that only provides values relative to Fresh hearts) were depleted by prolonged hypothermic storage to 24% ($n=4$) of pre-storage aerobic values. ATP content recovered rapidly upon Langendorff mode reperfusion and reached values equivalent to 70% of Fresh values after 5 min of reperfusion.

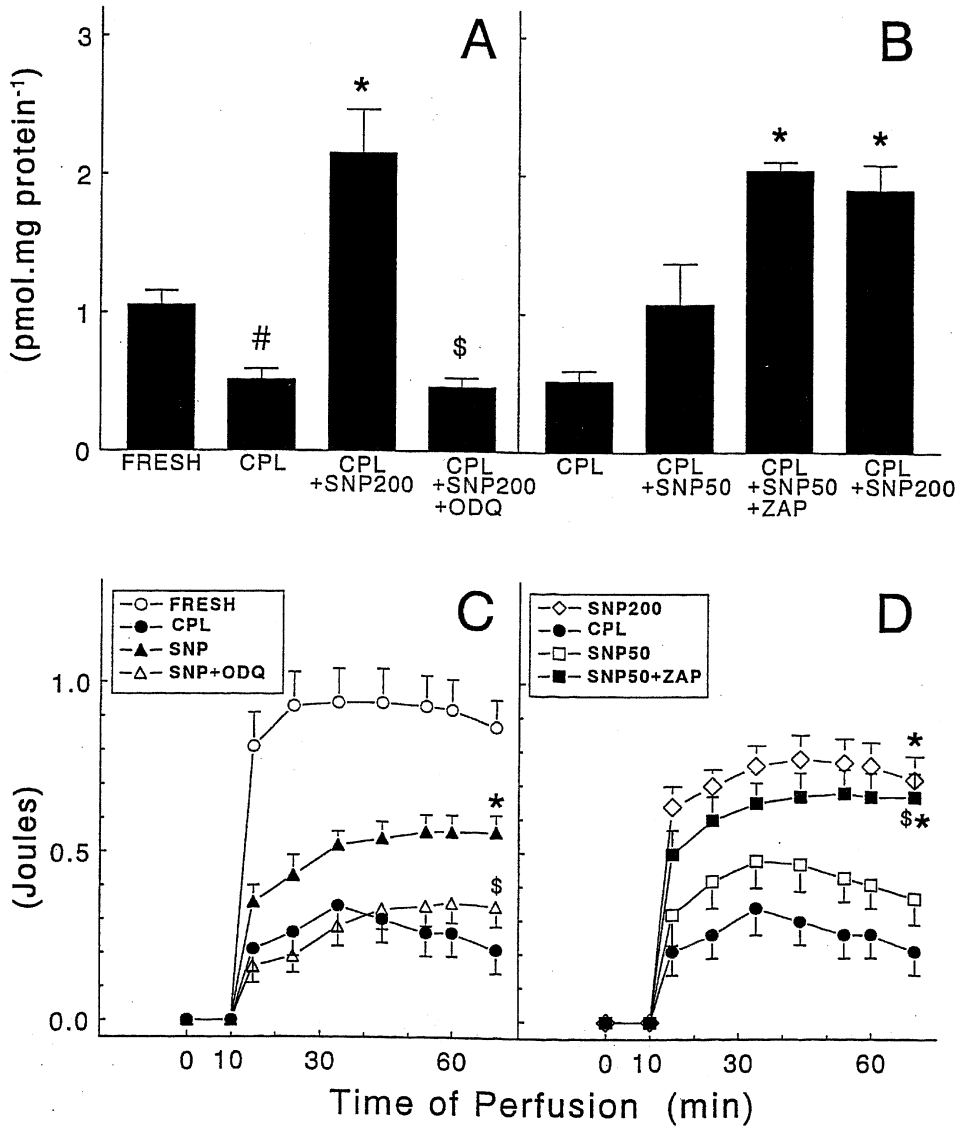

Figure 2 Content of cyclic GMP (A and B) and LV work (C and D) in Fresh and CPL hearts. (A) Content of cyclic GMP in Fresh ($n=6$), untreated CPL hearts ($n=6$) as well as CPL hearts treated only during Langendorff reperfusion with SNP alone (200 μM, $n=4$), or in the presence of ODQ (3 μM, SNP 200+ODQ, $n=4$). (B) Content of cyclic GMP in CPL hearts treated throughout storage and Langendorff reperfusion phases with SNP alone (50 μM, $n=4$) or in the presence of zaprinast (20 μM, SNP 50+ZAP, $n=4$) as well as with 200 μM SNP alone (SNP 200, $n=4$). (C) LV function during working mode reperfusion for Fresh ($n=6$) and untreated CPL hearts ($n=8$). Data are also shown for CPL hearts treated only during Langendorff reperfusion with SNP alone (200 μM, $n=8$) or in the presence of ODQ (3 μM, SNP+ODQ, $n=8$) (D) LV function during working mode reperfusion for hearts treated throughout arrest, storage and Langendorff mode and working mode reperfusion with SNP alone (50 μM, SNP 50, $n=8$) or in the presence of zaprinast (20 μM, SNP 50+ZAP, $n=8$) as well as with 200 μM SNP alone (SNP 200, $n=8$). #Indicates a significant difference between values in Fresh and untreated CPL hearts; *indicates a significant difference between untreated CPL and treated CPL groups and \$indicates a significant difference between CPL groups treated with SNP alone and those treated with SNP in combination with either ODQ or zaprinast.

Activities of eNOS and iNOS

In order to determine if the deficiency in cyclic GMP content was due to impaired NO production in the CPL heart, Ca^{2+}-dependent (eNOS) and Ca^{2+}-independent (iNOS) NOS activities (pmol min^{-1} mg $protein^{-1}$) were measured in Fresh ($n=6$) and untreated CPL hearts ($n=6$) frozen at the end of the 10-min period of Langendorff mode perfusion. When assayed at pH = 7.2 (Figure 3A), eNOS accounted for the majority of total NOS activity in Fresh (total: 2.41 ± 0.88; eNOS 2.41 ± 0.88) as well as CPL (total 1.92 ± 0.51; eNOS 1.90 ± 0.49) hearts. Both eNOS and iNOS activities were similar in Fresh and CPL hearts. However, if assays were performed at pH = 6.3 (Figure 3B) to mimic the intracellular pH of hearts at the onset of reperfusion (as determined by NMR spectroscopy), total NOS activity was reduced in both Fresh (1.02 ± 0.78) and CPL (0.64 ± 0.28) hearts relative to activities measured at pH = 7.2. Whereas the activity of iNOS was not significantly affected by the lower pH, eNOS activity was significantly depressed in both Fresh (0.59 ± 0.52) and CPL (0.43 ± 0.31) hearts.

Sensitivity of soluble guanylyl cyclase

The sensitivity of sGC to NO-induced stimulation, as assessed by the concentration of SNP required to elicit half-maximal activation (EC_{50}), was compared in Fresh ($n=6$) and untreated CPL hearts ($n=6$). When assayed at pH = 7.2 (Figure 3C), maximal sGC activity was not different between groups but the sensitivity to SNP in CPL hearts (93 ± 4 μM) was significantly greater than in Fresh (141 ± 19 μM) hearts. The activation of sGC by the concentration of SNP used in the perfusion experiments (200 μM) was significantly greater in untreated CPL than in Fresh hearts. The maximal activity of sGC was not affected by acidic pH (Figure 3D) and again, comparison of the EC_{50} values for SNP-induced activation of sGC indicated that the sensitivity of sGC was significantly increased in CPL (134 ± 9 μM) relative to Fresh (216 ± 28 μM) hearts.

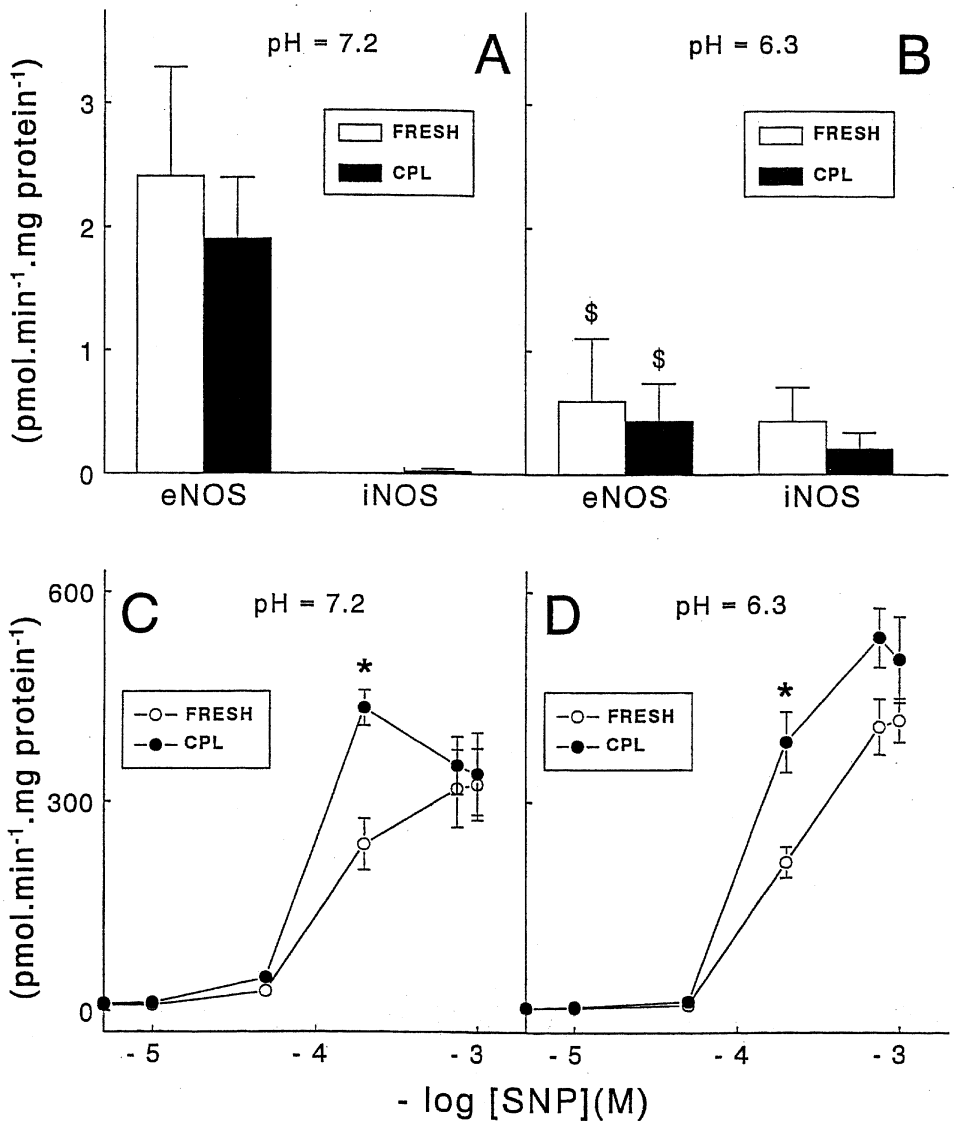

Figure 3 Activities of NOS (A and B) in Fresh and untreated CPL hearts frozen at the end of Langendorff perfusion. Values are shown for rates of citrulline production in Fresh ($n=6$) and untreated CPL hearts ($n=6$) when assayed at normal pH of 7.2 (A) and at pH = 6.3 (B). Sensitivity of sGC (C and D) in Fresh ($n=6$) and untreated CPL hearts ($n=6$) to SNP (0–1000 μM) at normal assay pH of 7.2 (C) and at pH = 6.3 (D). $Indicates a significant difference between NOS activities at pH = 7.2 and pH = 6.3. *Indicates a significant difference between sGC activities in Fresh and CPL hearts.

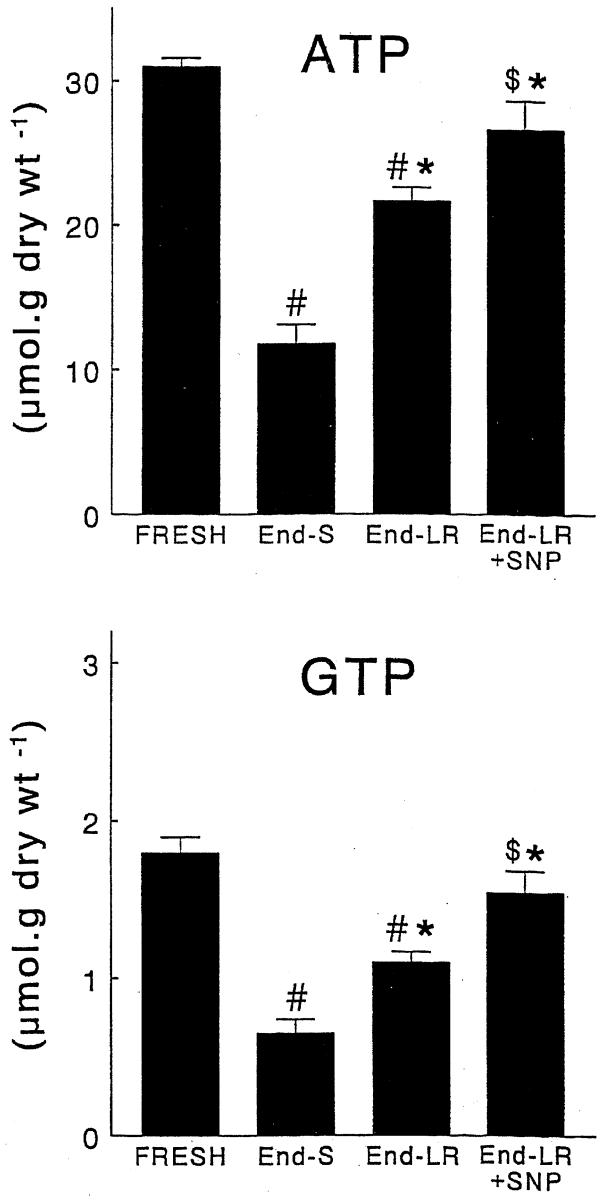

Figure 4 Contents of ATP and GTP in Fresh and CPL hearts. Values were measured in Fresh hearts frozen at the end of Langendorff perfusion (Fresh, $n=6$), in CPL hearts after arrest and storage (End-S, $n=6$), in hearts frozen after arrest, storage and Langendorff reperfusion (End-LR, $n=10$), and in hearts treated with SNP (200 μM) during arrest, storage and Langendorff reperfusion (End-LR + SNP, $n=4$). #Indicates a significant difference from Fresh; *indicates a significant difference from End-S and $indicates a significant difference between the SNP-treated group and the corresponding untreated CPL group at the end of Langendorff mode reperfusion.

Content of high-energy phosphates

The contents of ATP and GTP in CPL hearts were measured at the end of the period of hypothermic storage immediately prior to the onset of Langendorff mode reperfusion ($n=6$) as well as at the end of the 10-min period of Langendorff mode reperfusion in the absence ($n=10$) or presence ($n=4$) of SNP (200 μM) and compared with values obtained in Fresh hearts after 10-min Langendorff mode perfusion ($n=6$) (Figure 4). ATP content, determined by HPLC, was depleted in untreated CPL hearts to 38% of the content of Fresh hearts. Similarly, NMR spectroscopy (that only provided relative values) indicated that ATP contents were depleted by prolonged hypothermic storage to 24% ($n=4$) of values in Fresh hearts and that ATP content recovered rapidly upon Langendorff mode reperfusion to values equivalent to 70% of Fresh hearts. Moreover, treatment of CPL hearts with SNP (200 μM) during cardioplegic arrest, storage and the initial 10-min period of Langendorff mode reperfusion, that improved the subsequent recovery of mechanical function, showed a further increase in ATP content to values similar to those in Fresh hearts (Figure 4).

As described for ATP, the content of GTP was depleted in CPL hearts at the end of the storage period to 36% of aerobic values (Figure 4). GTP content increased by 70% during Langendorff mode reperfusion and recovery was accelerated by 138% in SNP-treated hearts to values similar to those observed in Fresh hearts. Values for other metabolites are presented in Table 3.

Activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH)

To determine whether the ability of hearts to produce superoxide anions, and hence reduce NO bioavailability, was altered by cardioplegic arrest and prolonged storage, the activities of XO and XDH in Fresh ($n=4$) and untreated CPL hearts ($n=4$) were compared (Figure 5A). After 10 min Langendorff mode reperfusion, activities of both XO and XDH in untreated CPL hearts were 302 and 200% of values in Fresh hearts.

LV compliance

Increases in LV end diastolic pressure (LVEDP) in response to graded increases in LV volume was used as an index of LV compliance in untreated ($n=6$) and SNP-treated ($n=6$) CPL hearts (Figure 5B). During Langendorff mode reperfusion, the slope of the relationship between LVEDP and LV volume was significantly lower in hearts exposed to SNP (200 μM). This indicates that SNP improved LV compliance during early Langendorff mode reperfusion.

Table 3 Metabolites determined in hearts frozen at selected stages of the perfusion protocol

Perfusion groups (n)	*Creatine*	*Creatine-P*	*ATP*	*ADP*	*AMP*	*Adenosine*	*GTP*
				μmol g dry weight^{-1}			
Fresh (6)	129.9±6.1	25.5±7.9	31.0±0.6	18.9±1.1	2.6±0.4	0.13±0.01	1.80±0.10
End-storage (6)	192.0±3.5	10.6±1.0	11.8±1.35#	24.6±1.0	39.9±3.6#	0.16±0.01	0.65±0.09#
End-LR (10)	128.7±5.4	19.2±0.6	21.7±1.0#*	16.1±0.8	8.6±0.7*	0.49±0.04*	1.10±0.07#*
End-LR+SNP (4)	116.7±13.7	18.1±0.4	26.6±2.0*$	14.8±0.2	5.2±1.6*	0.24±0.02$	1.54±0.14*$

Values are mean±s.e.mean. Significant differences from Fresh hearts frozen after 10 min Langendorff perfusion and CPL hearts frozen after hypothermic storage (End-storage) are indicated by # and *, respectively, whereas differences between groups frozen after 10-min Langerdorff reperfusion in the absence (End-LR) and presence of SNP (End-LR+SNP) are indicated by $.

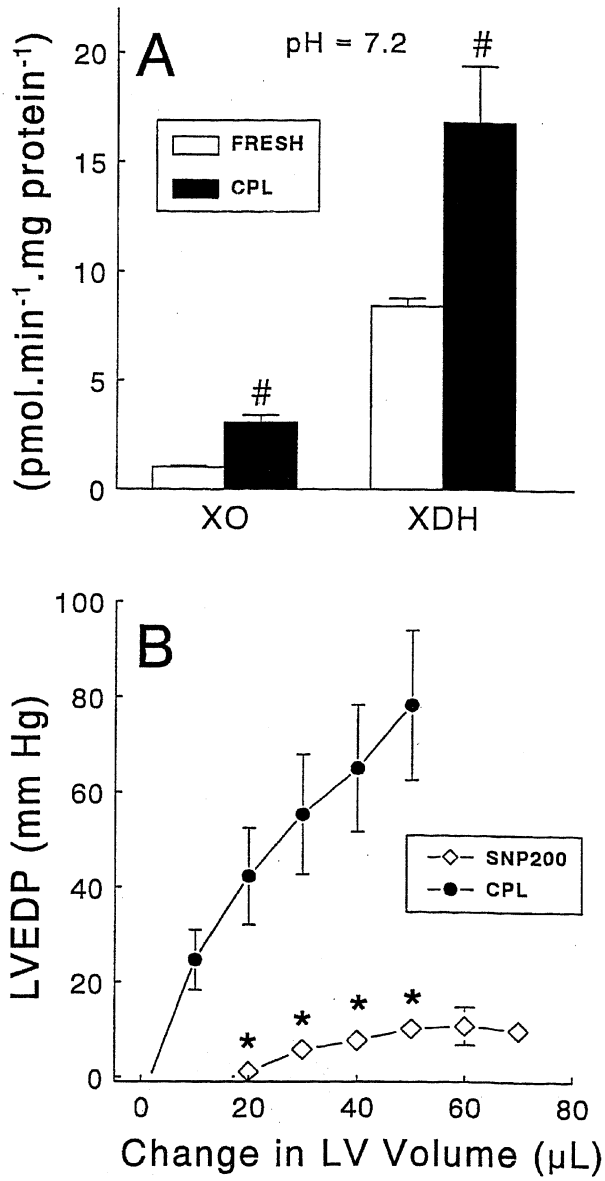

Figure 5 (A) Activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) measured in Fresh ($n=4$) and untreated CPL hearts ($n=4$) frozen at the end of the Langendorff perfusion and assayed at pH = 7.2. #Indicates a significant difference between Fresh and untreated CPL hearts. (B) Assessment LV compliance of CPL hearts either in the absence (CPL, $n=6$) or presence of SNP (200 μM, $n=6$). *Indicates a significant difference between untreated and SNP-treated CPL hearts.

Discussion

This study has compared the integrity of the NO/cyclic GMP pathway in Fresh and CPL hearts. We determined that in hearts, which had been subjected to cardioplegic arrest and prolonged (8 h) hypothermic (3°C) storage, there is a relative deficiency in cyclic GMP content and a poor recovery of mechanical function. When this cyclic GMP deficiency is corrected by the addition of the exogenous NO-donor, SNP, the recovery of the mechanical function upon subsequent working mode reperfusion is significantly enhanced. Inhibition of the beneficial effects of SNP on mechanical function by the sGC inhibitor, ODQ, and their potentiation by the cyclic GMP phosphodiesterase inhibitor, zaprinast, provide direct confirmation of the role of cyclic GMP in the cardioprotective action of SNP. Importantly, this beneficial effect of SNP results from a direct action on the cardiomyocyte as the perfusate was devoid of blood cells and the rate of coronary perfusion was unaltered. Moreover, this beneficial effect of SNP on mechanical function is manifest only on the stored and reperfused heart as SNP had no effects in Fresh hearts (Table 2).

In untreated CPL hearts, we found that the deficiency in NO biosignalling is not due to a reduction in the sensitivity of sGC to activation by NO donors, but may arise in response to a number of other mechanisms including acidosis-induced inhibition of NOS activity in the early reperfusion period, reduced availability of GTP for cyclic GMP generation by sGC and/or enhanced inactivation of NO by an accelerated superoxide anion production due to storage- and reperfusion-induced stimulation of XO/XDH activity. These findings provide rationales for the development of novel strategies to prevent the deficiency in NO bioavailability and so improve the recovery of mechanical function of the transplanted donor heart.

Cardioprotective efficacy of NO/cyclic GMP signalling

The cardioprotective efficacy of NO in models of ischaemia and reperfusion remains controversial (Curtis & Pabla, 1997) and evidence has been presented that indicate that NO is protective (Pinsky *et al*., 1994; Nakanishi *et al*., 1995; Lefer & Lefer, 1996; Lefer, 1995; Ma *et al*., 1993) as well as detrimental (Pabla & Curtis, 1996; Schulz & Wambolt, 1995; Yasmin *et al*., 1997; Schultz *et al*., 1997). In models of cardioplegic arrest and prolonged hypothermic storage, earlier studies have demonstrated that the provision of exogenous NO donors improves the recovery of post-ischaemic mechanical function (Lefer, 1995), but the mechanisms underlying the cardioprotective actions have not been elucidated. As the beneficial effects of SNP are antagonized by ODQ, a selective inhibitor of sGC (Moro *et al*., 1996), the cardioprotective mechanism has been suggested to involve elevation of cyclic GMP (Ali *et al*., 1998). This study extends our previous observations and provides direct confirmation that, relative to Fresh hearts, hearts subjected to cardioplegic arrest and prolonged hypothermic storage are indeed deficient in cyclic GMP content. Moreover, correction of this deficiency in cyclic GMP with either SNP or SNP in combination with an inhibitor of cyclic GMP-dependent phosphodiesterase, zaprinast (Wood & Owen, 1989), enhances the recovery of post-ischaemic LV work whereas the inhibitor of sGC, ODQ (Moro *et al*., 1996), prevents the SNP-induced cardioprotection and the associated increase in cyclic GMP. It should be noted that zaprinast *per se* did not enhance cyclic GMP content or enhance recovery of myocardial mechanical function. These effects indicate that basal sGC activity was likely low and that the deficiency in cyclic GMP content is unlikely to arise from greater catabolism of cyclic GMP in response to an enhanced activity of cyclic GMP-dependent phosphodiesterase. However, as the contents of cyclic AMP are similar in Fresh hearts, in untreated CPL hearts and in SNP-treated CPL hearts, the deficiency in cyclic GMP is specific and is not simply due to a non-specific loss of intracellular cyclic nucleotides. Moreover, alterations in the post-ischaemic recovery of mechanical function are not related to cyclic AMP content and the well-established role of cyclic AMP as a mediator of positive inotropic responses. These results clearly identify cyclic GMP deficiency as an important determinant of post-ischaemic recovery and support the use of exogenous NO donors as cardioprotective agents.

Cardioprotective mechanisms of cyclic GMP

While a lack of NO may contribute to a positive feedback loop in which diminished levels of NO allow platelet and neutrophil accumulation that causes further endothelial dysfunction and further platelet and neutrophil accumulation (Egdell *et al*., 1994), such a mechanism cannot be operative in the crystalloid, cell-free perfusion model. Moreover, a lack of SNP-induced increases in coronary perfusion indicates that the cardioprotective mechanism does not involve the coronary circulation (Ali *et al*., 1998). In addition, the enhanced recovery of mechanical function in response to SNP is not simply due to a positive inotropic effect, as SNP had no effects on any of the parameters of mechanical function in aerobic working hearts.

A plausible mechanism for the beneficial action of SNP concerns LV diastolic function. While depression of cyclic GMP content exerts cardiodepressant effects (Kojda *et al*., 1997), an enhanced LV relaxation, possibly mediated by a reduction in L-type calcium current (Levi *et al*., 1994) or activation of K_{ATP} channels (Richard *et al*., 1995) has been reported (Paulus *et al*., 1994) in response to NO donors and cyclic GMP, with more marked effects occurring in post-ischaemic heart. Improvement in LV compliance as a mechanism by which SNP and the NO/cyclic GMP pathway contributes to cardioprotection is suggested by our LV pressure-volume data indicating that SNP-treated CPL hearts have an enhanced ability to relax in response to increases in intraluminal pressure. Such an action corrects the impairment in LV diastolic filling of the reperfused heart and so improves the ability of the reperfused working heart to perform work. Interestingly, cyclic GMP has been shown to influence myocardial glucose uptake (Depre *et al*., 1998) and to reduce indirect indices of myocardial glycolysis (Laustiola *et al*., 1983). Such effects require further investigation as they may improve the coupling between glycolysis and glucose oxidation, attenuate proton production and acidosis (Finegan *et al*., 1996) and so reduce ischaemia-induced Ca^{2+} overload and diastolic dysfunction. These effects also improve the efficiency of energy utilization in the post-ischaemic heart (Liu *et al*., 1996) and thereby contribute to the accelerated recovery of ATP and GTP as well as LV mechanical function.

Impaired production of NO as cause of cyclic GMP deficiency

The deficiency in cyclic GMP content in the untreated CPL heart, in the absence of any apparent increase in cyclic GMP-dependent phosphodiesterase, suggests that NO availability was attenuated. While one mechanism may be related to impaired NO production due to alteration of myocardial NOS activity, no differences were noted between NOS activities in Fresh and untreated CPL hearts when assays were performed at the standard assay pH of 7.2. However, NOS activity decreases 2 fold between pH 7.4 and 6.7 (equivalent to mild ischaemia) (Fleming *et al*., 1994). Since measurement of intracellular pH by NMR spectroscopy in untreated CPL hearts (perfused under identical conditions to those used for studies of mechanical function and enzyme activities) indicated that cytosolic pH was 6.3 immediately prior to reperfusion, NOS activities were also measured under similar acidic conditions. At pH = 6.3, Ca^{2+}-dependent NOS activity was substantially lower suggesting that ischaemic acidosis-induced inhibition of NOS activity during the first few minutes of reperfusion may contribute to the deficiency in NO in the CPL heart. Recent evidence indicates that MLA (Zhao *et al*., 1997) and the second window of protection (SWOP) induced by ischaemic preconditioning (Qiu *et al*., 1997) cause a low-level induction of NOS, that in effect stimulates endogenous NO production and so may ultimately correct the post-ischaemic impairment of myocardial NO biosignalling.

Impaired sGC sensitivity as cause of cyclic GMP deficiency

One important transduction pathway for NO involves the activation of sGC that catalyzes the production of cyclic GMP from GTP (Murad *et al*., 1979), but its activity in the post-storage heart has not been examined. In order to determine if the deficiency of cyclic GMP in untreated CPL hearts was due to decreased sensitivity or inactivation of sGC, the EC_{50} for SNP-induced activation of sGC was compared in Fresh and untreated CPL hearts. Maximal activities of this enzyme were similar in Fresh and CPL hearts, but the EC_{50} of SNP in untreated CPL hearts was lower indicating that the deficiency in cyclic GMP in CPL hearts cannot be explained by impaired activity or sensitivity of sGC. The activity of sGC was not influenced by assay pH. Interestingly, the sensitivity of sGC to SNP was greater in CPL hearts than in Fresh hearts. As sensitization of sGC has been noted previously in response to agonist removal (Davis *et al*., 1997), this result provides additional evidence that NO bioavailability was reduced in CPL hearts. The inability of zaprinast *per se* to increase cyclic GMP content further supports the notion that CPL hearts have a relative lack of NO availability and depressed sGC activity.

It should be noted that sGC activity was assayed under controlled conditions of substrate (GTP) availability where the concentration of GTP (200 μM) is greater than its K_m (130 μM) for the enzyme (Waldman *et al*., 1991). However, assays of myocardial GTP content revealed that there is marked depletion of GTP in the untreated CPL heart that could also contribute to lower sGC activity and impaired cyclic GMP production.

Deficiency in NO biosignalling due to increases in NO inactivation

Impaired NO bioavailability, in association with enhanced levels of reactive oxygen species, particularly superoxide, occur in several pathologies including diabetes, atherosclerosis, hypertension as well as ischaemia reperfusion injury (Oury *et al*., 1996). Through its chemical interaction with NO, superoxide impairs NO bioavailability and reduces endothelium-dependent vasodilatation (Laight *et al*., 1998). Similar mechanisms have been proposed to operate within cardiac muscle where the interaction between superoxide and NO, not only limits NO bioavailability, but also increases the production of peroxynitrite anion (Yasmin *et al*., 1997) that, when protonated, dissociates to form hydroxy radical and nitrogen dioxide. Both of these products are potent oxidizing agents and cause tissue injury. As an index of the capacity of untreated CPL hearts and Fresh hearts to synthesize superoxide, the activities of xanthine dehydrogenase (XDH) and xanthine oxidase (XO) were assayed. The activities of both enzymes were significantly elevated in CPL hearts indicating that hearts subjected to cardioplegic arrest, prolonged hypothermic storage and reperfusion have an enhanced potential for superoxide production, even in the absence of blood-borne cellular elements. While these data provide evidence of an additional mechanism in rat heart that may account for impaired NO bioavailability, the relevance to the human heart with low XO activity is uncertain.

Conclusions

Relative to fresh hearts, CPL hearts have a deficiency in cyclic GMP content and a poor recovery of LV work and correction of this deficiency with SNP improves LV work and LV compliance. The role of sGC was confirmed by the abilities of ODQ and zaprinast to inhibit and potentiate, respectively, the beneficial actions of SNP on LV mechanical function and cyclic GMP content. In untreated CPL hearts, the deficiency in NO biosignalling is not due to a reduction in the sensitivity of sGC, but may arise due to acidosis-induced inhibition of NOS activity, reduced availability of GTP for cyclic GMP generation by sGC and/or enhanced inactivation of NO by superoxide anion generated by CPL-induced stimulation of XO/XDH activity. These findings reveal the nature of the deficiency of the NO/cyclic GMP pathway in hearts subjected to cardioplegia and prolonged hypothermic storage and identify targets for novel strategies to enhance NO biosignalling and so extend the safe ischaemic time as well as improve the mechanical function of the transplanted heart.

This work was supported by a grant-in-aid from MRC (Canada). We thank Dr R. Schulz for expert assistance with the development of the assays of NOS, XO and XDH activities.

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(Received March 29, 1999
Revised July 19, 1999
Accepted July 29, 1999)