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The mechanism of cardioprotection by S-nitrosoglutathione monoethyl ester in rat isolated heart during cardioplegic ischaemic arrest

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1 This study was designed (i) to assess the effect of S-nitrosoglutathione monoethyl ester (GSNO-MEE), a membrane-permeable analogue of S-nitrosoglutathione (GSNO), on rat isolated heart during cardioplegic ischaemia, and (ii) to monitor the release of nitric oxide (·NO) from GSNO-MEE in intact hearts using endogenous myoglobin as an intracellular ·NO trap and the hydrophilic N-methyl glucamine dithiocarbamate-iron (MGD-Fe^{2+}) complex as an extracellular ·NO trap.

2 During aerobic perfusion of rat isolated heart with GSNO-MEE (20 μmol l^{-1}), there was an increase in cyclic GMP from 105 ± 11 to 955 ± 193 pmol g^{-1} dry wt. ($P<0.05$), and a decrease in glycogen content from 119 ± 3 to 96 ± 2 μmol g^{-1} dry wt. ($P<0.05$), and glucose-6-phosphate concentration from 258 ± 22 in control to 185 ± 17 nmol g^{-1} dry wt. ($P<0.05$). During induction of cardioplegia, GSNO-MEE caused the accumulation of cyclic GMP (100 ± 6 in control *vs.* 929 ± 168 pmol g^{-1} dry wt. in GSNO-MEE-treated group, $P<0.05$), and depletion of glycogen from 117 ± 3 to 103 ± 2 μmol g^{-1} dry wt. ($P<0.05$) in myocardial tissue.

3 Inclusion of GSNO-MEE (20 μmol l^{-1}) in the cardioplegic solution improved the recovery of developed pressure (46 ± 8 *vs.* 71 ± 3% of baseline, $P<0.05$), and rate-pressure product from 34 ± 6 to 63 ± 5% of baseline ($P<0.05$), and reduced the diastolic pressure during reperfusion from 61 ± 7 in control to 35 ± 5 mmHg ($P<0.05$) after 35 min ischaemic arrest. GSH-MEE (20 μmol l^{-1}) in the cardioplegic solution did not elicit the protective effect.

4 During cardioplegic ischaemia, GSNO-MEE (20–200 μmol l^{-1}) induced the formation of nitrosylmyoglobin (MbNO), which was detected by electron spin resonance (ESR) spectroscopy. Inclusion of MGD-Fe^{2+} (50 μmol l^{-1} Fe^{2+} and 500 μmol l^{-1} MGD) in the cardioplegic solution along with GSNO-MEE yielded an ESR signal characteristic of the MGD-Fe^{2+}-NO adduct. However, the MGD-Fe^{2+} trap did not prevent the formation of the intracellular MbNO complex in myocardial tissue. During aerobic reperfusion, denitrosylation of the MbNO complex slowly occurred as shown by the decrease in ESR spectral intensity. GSNO-MEE treatment did not affect ubisemiquinone radical formation during reperfusion.

5 GSNO-MEE (20 μl l^{-1}) treatment elevated the myocardial cyclic GMP during ischaemia (47 ± 3 in control *vs.* 153 ± 34 pmol g^{-1} dry wt. after 35 min ischaemia, $P<0.05$). The cyclic GMP levels decreased in the control group during ischaemia from 100 ± 6 after induction of cardioplegia to 47 ± 3 pmol g^{-1} dry wt. at the end of ischaemic duration.

6 Glycogen levels were lower in GSNO-MEE (20 μmol l^{-1})-treated hearts throughout the ischaemic duration (26.7 ± 3.1 in control *vs.* 19.7 ± 2.4 μmol g dry^{-1} wt. in GSNO-MEE-treated group at the end of ischaemic duration), because of rapid depletion of glycogen during induction of cardioplegia. During ischaemia, the amounts of glycogen consumed in both groups were similar. Equivalent amounts of lactate were produced in both groups (148 ± 4 in control *vs.* 141 ± 4 μmol g^{-1} dry wt. in GSNO-MEE-treated group after 35 min in ischaemia).

7 The mechanism(s) of myocardial protection by GSNO-MEE against ischaemic injury may involve preischaemic glycogen reduction and/or elevated cyclic GMP levels in myocardial tissue during ischaemia.

Keywords: Nitric oxide; nitrosothiols; electron spin resonance; glycogen; cyclic GMP

Introduction

Nitrovasodilators have long been used to alleviate myocardial ischaemic syndromes, but only recently has it been suggested that their effects on vascular tissues and blood elements are mediated by ·NO (Feelish & Noack, 1987; Ahlner *et al.*, 1991). This discovery is pivotal for rational design of a new class of mechanism-based anti-ischaemic drugs that can release nitric oxide in a controlled fashion. ·NO donors show a broad spectrum of pharmacological activities toward cardiovascular disorders. ·NO donors prevent platelet aggregation, extravascular albumin leakage, leucocyte rolling and adhesion to a vascular wall, and protect against coronary artery thrombosis and reocclusion after thrombolysis (Siegfried 1992; Gaboury *et al.*, 1993; Kurose *et al.*, 1993; Yao *et al.*, 1995). ·NO donors also inhibit the proliferation of smooth muscle and endothelial cells, prevent neointima formation and vascular reocclusion after transluminal coronary angioplasty (Yang *et al.*, 1994; De Meyer *et al.*, 1995; Grovers *et al.*, 1995), and protect the heart against ischaemia-reperfusion injury *in vivo* and in models of cardioplegic ischaemic arrest (Siegfried *et al.*, 1992; Pinsky *et al.*, 1994; Konorev *et al.*, 1995b).

Previously, we investigated the myocardial protection by S-nitrosoglutathione (GSNO) in a model of cardioplegic ischaemic arrest. GSNO is a physiologically relevant ·NO

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donor that is stable in aqueous solutions and releases ·NO upon interaction with redox metal ions (Mathews & Ker, 1993; Park *et al.*, 1993). However, the mechanism of targeting of ·NO inside myocardial cells is unclear because GSNO is unlikely to permeate the cells, as does GSH (Martensson & Meister, 1989).

In the present study, we have used S-nitrosoglutathione monoethyl ester (GSNO-MEE), a membrane-permeable lipophilic analogue of GSNO (Figure 1). The glutathione esters, unlike glutathione, are effectively transported into cells and hydrolyzed to release glutathione intracellularly (Anderson *et al.*, 1990). Glutathione esters were shown to protect against glutathione deficiency, radiation, and xenobiotic-induced toxicity; whereas, exogenous glutathione was ineffective in these conditions (Astor *et al.*, 1988; Martensson & Meister, 1989; Anderson *et al.*, 1990). In this study, we investigated the ability of GSNO-MEE, an ester of GSNO, to protect against cardioplegic ischaemic injury. Using myoglobin as an intracellular ·NO trap, we also investigated the formation of nitrosylmyoglobin (MbNO) in the ischaemic hearts. The MGD-Fe^{2+} complex, a hydrophilic ·NO scavenger, was used to trap ·NO released from GSNO-MEE extracellularly (Figure 1).

Methods

Isolated heart preparation

Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, U.S.A.), 275–325 g body wt. maintained on a standard diet, were used for this study. Rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and heparinized via the left femoral vein (250 iu kg^{-1}). Each heart was excised rapidly and placed in perfusion medium. Within 30 s, the aorta was attached to a stainless steel cannula, and the heart was perfused at 37°C by the non-recirculated method of Langendorff. To maintain the temperature constant during perfusion, the heart was immersed in the perfusion medium present in a heated heart chamber. The hearts beat spontaneously and were perfused at a constant pressure equivalent to 12 kPa (90 mmHg). The perfusion medium was KHB, having the following composition (in mmol l^{-1}): NaCl 118.5, $NaHCO_3$ 25, KCl 4.8, $MgSO_4$ 1.2, glucose 11.1, $CaCl_2$ 1.8 (pH 7.4 when gassed with 95% O_2 and 5% CO_2). St Thomas' Hospital cardioplegic solution (STH) was used as a cardioplegic solution with the following composition (in mmol l^{-1}) NaCl 110, $NaHCO_3$ 10, KCl 16, $MgCl_2$ 16 and $CaCl_2$ 1.2. The pH of the solution was titrated to 7.8 with HCl immediately before use.

Cardiac function

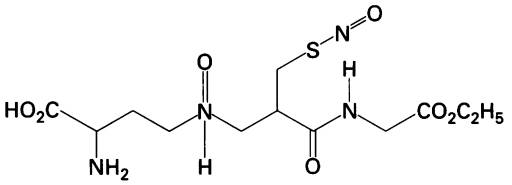
A saline-filled latex balloon was inserted into the left ventricle through the mitral valve and secured in place with a ligature. The balloon was connected by a rigid saline-filled catheter to a pressure transducer for the measurement of the left ventricular pressure and heart rate. The transducer output was amplified by a universal signal conditioner (Gould, Cleveland, OH, U.S.A.) and was recorded on an analog chart recorder (Astromed 9500, Providence, RI, U.S.A). The intraventricular balloon was inflated at the beginning of each experiment until the diastolic pressure was 3 mmHg. The rate pressure product (RPP) was determined as the product of heart rate per second and developed pressure. Coronary flow rate (ml min^{-1}) was measured by collecting the perfusate leaving the right heart into a graduated cylinder.

Myocardial cyclic GMP

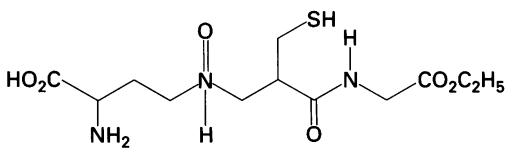
A 5% homogenate of freeze-clamped myocardial tissue was extracted in 50 mmol l^{-1} sodium acetate (pH 4.0) for 30 min at 0°C and was centrifuged at 4000 × g for 10 min at 4°C. The supernatant was assayed for guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content by an ELISA kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) as described previously (Konorev, 1995a).

Myocardial metabolites

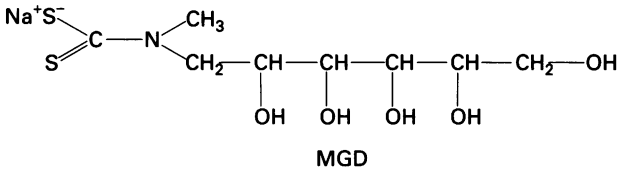
Hearts were freeze-clamped with the clamp precooled in liquid nitrogen and stored in liquid nitrogen until assay. Homogenization was performed in 1 N ice-cold perchloric acid. An



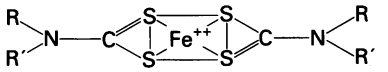
S-nitrosoglutathione monoethyl ester
(GSNO-MEE)



Glutathione monoethyl ester
(GSH-MEE)

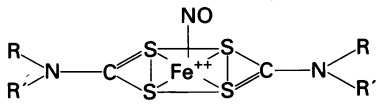


MGD



R = CH_3 R′ = $CH_2(CHOH)_4CH_2OH$

MGD-Fe^{2+}



R = CH_3 R′ = $CH_2(CHOH)_4CH_2OH$

MGD-Fe^{2+}-NO

Figure 1 Chemical structures of selected chemicals.

aliquot of homogenate was taken for glycogen measurement (Bergmeyer, 1974). The remaining homogenate was kept on ice for 30 min for extraction of metabolites and centrifuged at 2°C for 10 min at 3000 g. The supernatant was neutralized with 5 mol l^{-1} K_2CO_3 to pH 5–6 and centrifuged at 2°C for 10 min at 3000 *g* to remove potassium perchlorate. Neutralized extracts were used for enzymatic determination of myocardial metabolites (Bergmeyer, 1974).

Electron spin resonance (ESR) spectroscopy

ESR spectra from all samples were recorded at liquid nitrogen temperature on a Varian E-109 spectrometer at 9.5 GHz and 100 kHz field modulation. Magnetic field measurements were determined by using a Radiopan MJ-110 gaussmeters, and microwave frequency was measured with an EiP 200 frequency counter. The g-values were estimated from measurement of magnetic field and microwave frequency after correcting for the position of the gaussmeter probe. All spectra were obtained by signal averaging (5 scans).

Tissue Preparation for ESR

After selected intervals of aerobic perfusion, ischaemia, and reperfusion, as shown in the experimental protocols (see Figure 2), hearts were freeze-clamped between stainless steel tongs previously cooled to liquid nitrogen temperature. Frozen ventricular tissue was then chopped under liquid nitrogen with a stainless steel spatula to produce small fragments (2 mm cubes). These fragments were immediately transferred to the lumen of dewar flask that had been precooled to the temperature of liquid nitrogen. All flasks containing the processed heart tissue were then placed in the resonance chamber of the spectrometer. Samples prepared by this technique did not artefactually generate radical species (Baker *et al.*, 1988).

Reagents

Glutathione monoethyl ester (GSH-MEE) was synthesized according to Campbell & Griffith (1989). GSNO-MEE was prepared by nitrosylation of GSH-MEE according to Hart (1985). N-methyl glucamine dithiocarbomate (MGD) was synthesized as described by Shinobu *et al.* (1984). 'Baker analysed' high purity reagents (JT Baker, Philipsburg, NJ, U.S.A.) were used in the preparation of heart perfusion media. Perchloric acid was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Citrate lyase was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

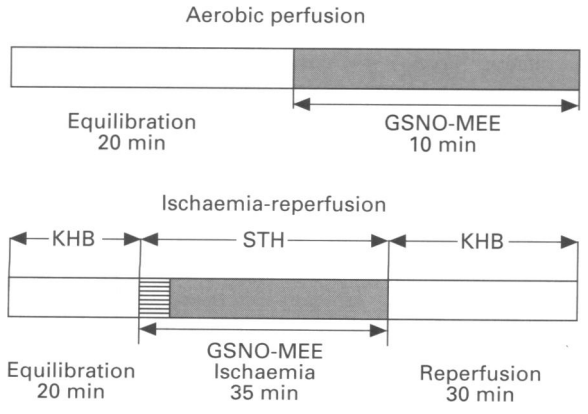


Figure 2 Experimental protocols: hatched area, 4 min induction of cardioplegia with St Thomas' Hospital cardioplegic solution (STH) to arrest the heart before ischaemia.

Preparation of MGD-Fe^{2+} complex

During preparation of MGD-Fe^{2+} complex, precautions were taken to prevent precipitation, which occurs upon oxidation of Fe^{2+} to Fe^{3+}. Freshly made solutions of $FeSO_4$ (2.5 mmol l^{-1}) and MGD (25 mmol l^{-1}) in deionized milliQ brand water were purged with nitrogen for 30 min. Immediately before use, equal aliquots of each solution were combined to form the yellowish-brown MGD-Fe^{2+} complex, which was infused via side arm to the heart perfusion line during induction of cardioplegia at the flow rate of 0.5 ml min^{-1}. The flow rate of STH during induction of cardioplegia was 12 ml min^{-1}, and the concentration of the MGD-Fe^{2+} in the perfusate was 50 μmol l^{-1} with the excess of MGD to prevent the precipitation of the complex in the cardioplegic solution.

Experimental protocols

Aerobic perfusion To study the effect of GSNO-MEE on aerobic myocardial metabolism, hearts were perfused with oxygenated Krebs-Henseleit buffer (KHB) at 37°C for a 20 min equilibration period at a constant flow of 15 ml min^{-1} (Figure 2). GSNO-MEE (20 μmol l^{-1}) was infused via a side arm for 10 min. GSNO-treated and control hearts (which were perfused with KHB only) were freeze-clamped at the end of experiments for cyclic GMP and metabolic measurements.

Ischaemia-reperfusion Hearts were perfused aerobically with KHB at 37°C for a 20 min equilibration period at constant pressure as described earlier. During this period, baseline measurements of left ventricular pressure and heart rate were taken. Cardioplegia was induced with a 4 min infusion of STH at 37°C. Because GSNO-MEE is photolabile, experiments were performed in subdued light and glassware was covered with aluminium foil. Fresh solutions of GSNO-MEE and GSH-MEE were kept on ice, and infused via a side arm at concentrations of 20 μmol l^{-1} during induction of cardioplegia. Hearts were then subjected to normothermic global ischaemia in STH for 35 min. After the ischaemic duration, hearts were reperfused with KHB for 30 min.

In separate experiments, hearts were freeze-clamped after induction of cardioplegia and after 10 or 35 min of cardioplegic ischaemia for cyclic GMP and metabolites measurements.

Statistical analysis

All data are presented as mean ± s.e.mean. Statistical analysis was performed initially by analysis of variance. Data were then subjected to Student's *t* test incorporating the Bonferroni correction for multiple comparisons. Significance was accepted at the $P<0.05$ level.

Ethical considerations

Animals used in this study received humane care in compliance with *Guiding Principles in the Care and Use of Animals* approved by the Council of the American Physiological Society and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science and published by the National Institutes of Health (NIH publ. 85-23, revised 1985).

Results

Effect of GSNO-MEE during aerobic perfusion of rat isolated heart

GSNO-MEE (20 μmol l^{-1}) did not change the contractile function of the rat isolated heart compared with control (data not shown). Data on cyclic GMP and myocardial metabolites

are shown in Table 1. GSNO-MEE induced a nine-fold increase in myocardial cyclic GMP. Reduction of the glycogen content in GSNO-MEE-treated hearts was associated with a decrease in glucose-6-phosphate (Glu-6-P) level. ATP and citrate levels were similar in both groups (Table 1).

Effect of GSNO-MEE during induction of cardioplegia

Induction of cardioplegia with STH resulted in cessation of contractile activity and relaxation of the hearts. The cyclic GMP content was nine fold higher in the GSNO-MEE-treated groups as compared to control (Table 2). A two fold increase in citrate levels was observed in both groups as compared with aerobic perfusion with KHB. Glycogen contents were significantly reduced in GSNO-MEE group compared with control. A decreasing trend, although not statistically significant, in Glu-6-P level was observed in the GSNO-MEE-treated hearts.

Effect of GSNO during cardioplegic Ischaemic Arrest - ESR studies

The ESR spectra of ischaemic control, GSNO-MEE (20 μmol l^{-1})-treated and GSNO-MEE (200 μmol l^{-1})-treated heart tissues are shown in Figure 3a–c. The ESR spectrum can be assigned to a mixture of species: ubisemiquinone or flavin semiquinone radical (g = 2.005, marked ●), reduced iron sulphur centre (g = 1.94) probably associated with the mitochondrial NADH or succinate dehydrogenase (marked △), and MbNO (marked □). It is clear that increasing the GSNO concentration leads to an increase in the signal intensity of MbNO (*cf.* Figures 3b and c). To substantiate that no significant ·NO is released during 30 min of cardioplegic ischaemia in control hearts, we used a water soluble dithiocarbamate (MGD-Fe^{2+}) complex as an exogenous ·NO scavenger. Figure 4b shows no evidence of formation of MGD-Fe^{2+}-NO complex in control hearts. However, when MGD-Fe^{2+} was added to GSNO-treated hearts, three lines in the $g \perp$ region corresponding to MGD-Fe^{2+}-NO complex ($a \perp^N$ = 13.4 G, $g \perp$ = 2.039 G) were obtained (lines marked ↓ in Figure 4d). Figure 4d also shows that a significant fraction of ·NO released from GSNO is trapped intracellularly by myoglobin, even in the presence of exogenous ·NO trap (*cf.* Figures 4c and d). These results suggest that myoglobin is an effective intracellular trap for ·NO.

Figure 5a–c shows ESR spectra of aerobically-perfused control, ischaemic, and aerobically-reperfused hearts. There was a sudden burst in formation of the ubisemiquinone radical (marked ○ in Figure 5c) during the first 2 min of reperfusion. Upon reperfusion of GSNO-treated hearts, MbNO signal still remained (Figure 5d). Inclusion of GSNO also did not significantly affect the signal intensity of the ubisemiquinone radical formed during reperfusion (Figure 5d).

Effect of GSNO-MEE on myocardial metabolites during cardioplegic ischaemic arrest

The cyclic GMP level was significantly higher in GSNO-MEE groups compared with control throughout the duration of ischaemia. However, the cyclic GMP decreased in all groups during ischaemia (Table 3). At the end of ischaemic period, the cyclic GMP was two fold lower in control and six fold lower in GSNO-MEE-treated hearts compared with baseline values after induction of cardioplegia (Table 2). The ATP content was

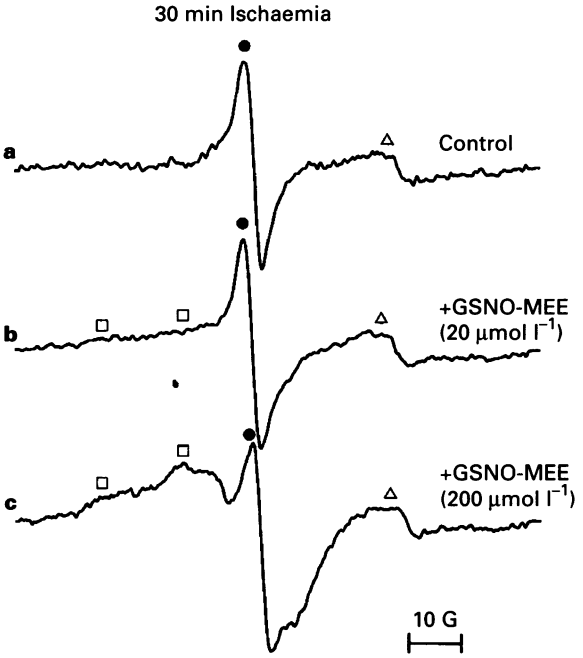


Figure 3 ESR spectra at 30 min of (a) ischaemic control, (b) ischaemic with GSNO (20 μmol l^{-1}), and (c) ischaemic with GSNO (200 μmol l^{-1}) rat hearts freeze-clamped and chopped at liquid nitrogen temperature prior to recording of the spectra at 77 K. Three hearts were used in each experimental group. Symbols: (●) secondary ubisemiquinone; (△) reduced iron-sulphur centre; (□) nitrosyl myoglobin. Spectrometer conditions: scan range, 100 G; scan time, 1 min; time constant, 0.064 s.

Table 1 Effect of GSNO-MEE (20 μmol l^{-1}) on myocardial metabolites and cyclic GMP during aerobic perfusion of rat isolated heart

Groups	*ATP* (μmol g^{-1} dry wt.)	*Glycogen*	*Glu-6-P* (nmol g^{-1} dry wt)	*Citrate*	*Cyclic GMP* (pmol g^{-1} dry wt)
Control (5)	23.9±0.5	119±3	258±22	579±70	105±11
GSNO-MEE (5)	24.0±0.4	96±2*	185±17*	559±58	955±193*

*$P<0.05$ as compared with control; (*n*), the number of hearts per group.

Table 2 Effect of GSNO-MEE (20 μmol l^{-1}) on myocardial metabolites and cyclic GMP after induction of cardioplegia with STH

Groups	*ATP* (μmol g^{-1} dry wt.)	*Glycogen*	*Citrate* (nmol g^{-1} dry wt.)	*Glu-6-P*	*Cyclic GMP* (pmol g^{-1} dry wt.)
Control (5)	23.7±0.5	117±3	1350±50	105±19	100±6
GSNO-MEE (5)	23.3±0.3	103±2*	1390±30	83±11	929±168*

*$P<0.05$ as compared with control; (*n*), the number of hearts per group.

similarly reduced in both groups during ischaemia. Glycogen was lower in GSNO-MEE hearts; however, at the beginning of ischaemia, glycogen levels in these hearts were already decreased. Thus, hearts from both groups used up similar amounts of glycogen during ischaemia and produced equivalent amounts of lactate. The citrate level was lower in the GSNO-MEE group after 10 min ischaemia. There was a decreasing but not statistically significant trend in Glu-6-P and citrate levels in this group after 35 min ischaemia.

Effect of GSNO-MEE on postischaemic functional recovery

Functional parameters of cardiac function after 20 min equilibration period (baseline values) are shown in Table 4. Reperfusion with oxygenated KHB buffer after 35 min cardioplegic ischaemia results in the resumption of contractile function (Figure 6). Recovery of contractile function was improved in the GSNO-MEE group, but not in the GSH-MEE-treated group. The recovery of coronary flow rate was increased in the GSNO-MEE-treated group to 67±4% of baseline, as compared to the control value of 46±5% of baseline ($P<0.05$). The diastolic pressure was elevated during reperfusion up to 61±7 and 56±6 mmHg in control and GSH-MEE-treated hearts, respectively. Inclusion of GSNO-MEE reduced the elevated diastolic pressure to 35±5 mmHg (Figure 6).

Discussion

·NO as an endogenous protective agent in myocardial tissue

Cardiac myocytes have been shown to possess constitutive ·NO synthase activity (Schulz *et al.*, 1992). Physiological responses of ventricular myocytes to muscarinic and β-adreno-

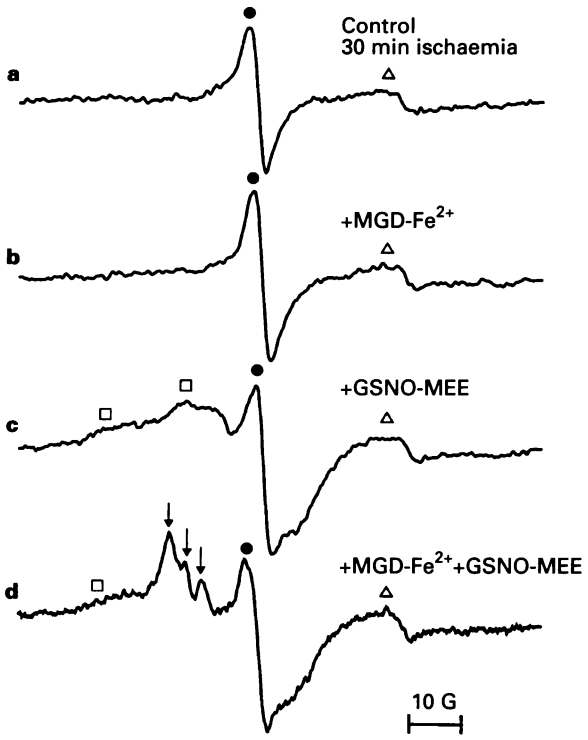


Figure 4 ESR spectra of (a) ischaemic, (b) ischaemic with MGD-Fe^{2+} complex, (c) ischaemic with 200 μmol l^{-1} GSNO, and (d) ischaemic with MGD-Fe^{2+} and GSNO (200 μmol l^{-1}) rat heart freeze-clamped and chopped at liquid nitrogen temperature prior to recording of the spectra at 77 K. Three hearts were used in each experimental group. Symbols: (●) secondary ubisemiquinone; (△) reduced iron-sulphur centre, (□) nitrosyl myoglobin, and ↓ line positions of MGD-Fe^{2+}-NO complex. Spectrometer conditions: microwave power, 0.2 mW; modulation amplitude, 3.2 G; gain, 6.3 × 10^4; scan range, 100 G; scan time, 1 min; time constant 0.064 s; microwave frequency, 9.2 GHz.

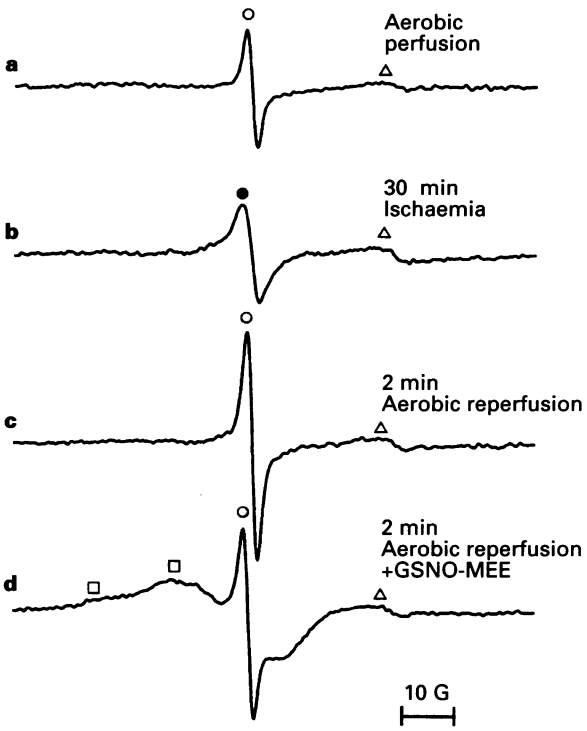


Figure 5 ESR spectra of (a) control, aerobically perfused, (b) 30 min ischaemic, and (c) 2 min aerobically reperfused rat heart freeze-clamped and chopped at liquid nitrogen temperature prior to recording of spectra at 77 K. (d) Same as (c), except hearts were aerobically reperfused for 2 min after 30 min ischaemic with GSNO-MEE (200 μmol l^{-1}). Three hearts were used for each experimental condition. Symbols: (○) ubisemiquinone radical; (●) secondary semiquinone; (△) reduced iron-sulphur centre, and (□) nitrosyl myoglobin. Spectrometer conditions: microwave power, 0.2 mW; modulation amplitude, 3.2 G; gain, 6.3 × 10^4; scan range 100 G; scan time, 1 min; time constant, 0.064 s.

Table 3 Effect of GSNO-MEE (20 μmol l^{-1}) on myocardial metabolites and cyclic GMP during cardioplegic ischaemia

Groups	*ATP*	*Glycogen* (μmol g^{-1} dry wt.)	*Glu-6-P*	*Lactate*	*Citrate* (nmol g^{-1} dry wt.)	*Cyclic GMP* (pmol g^{-1} dry wt.)
			10 min ischaemia			
Control (5)	19.0±0.8	104±6	1.38±0.17	42±2	930±43	78±10
GSNO-MEE (6)	19.5±0.5	88±3*	1.40±0.09	42±1	772±45*	819±83*
			35 min ischaemia			
Control (5)	5.8±0.3	26.7±3.1	3.81±0.45	148±4	702±88	47±3
GSNO-MEE (5)	5.0±0.5	19.7±2.4	2.96±0.14	141±4	534±62	153±34*

*$P<0.05$ as compared with control; (*n*), the number of hearts per group.

Table 4 Baseline functional parameters of rat isolated hearts before induction of cardioplegia

Groups	*CFR*	*HR*	*Dev P*	*RPP*
Control (5)	16.4±0.7	296±19	129±7	631±27
GSNO-MEE (5)	16.6±0.2	296±7	122±9	599±39
GSH-MEE (5)	16.4±0.2	298±24	125±5	618±36

CFR (ml min^{-1}), coronary flow rate; HR (beats min^{-1}), heart rate; Dev P (mmHg), developed pressure; RPP (mmHg × s), rate-pressure product. Diastolic pressure was set at 3 mmHg in all hearts.

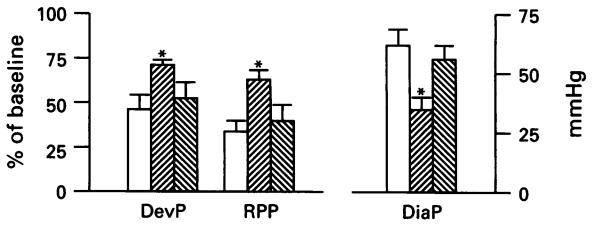


Figure 6 Effect of GSNO-MEE (20 $\mu mol l^{-1}$) and GSH-MEE (20 $\mu mol l^{-1}$) on the functional recovery of rat isolated hearts after 35 min cardioplegic ischaemic arrest. Recovery of left ventricular developed pressure (DevP, percentage of baseline), RPP (percentage of baseline), and diastolic pressure (DiaP, mmHg) after 30 min reperfusion are shown. □, control; ▨, GSNO-MEE; ▧, GSH-MEE. *$P<0.05$ compared with control, $n=5$ hearts per group.

ceptor stimulation are partially mediated by ·NO (Balligand *et al.*, 1993; Hare *et al.*, 1995). ·NO has been shown to induce transcription of the heat-shock protein HSP70, which renders the myocardial tissue resistant to ischaemic episodes (Malyshev *et al.*, 1995). Vegh *et al.* (1992) have suggested a protective role of ·NO during myocardial preconditioning against ischaemia-induced arrhythmias, as ·NO synthase inhibitors antagonize this cardioprotection. Also, myocardial protection by bradykinin, acetylcholine, and angiotensin-converting enzyme inhibitors against ischaemic injury was shown to be mediated by ·NO release, which was counteracted by ·NO synthase inhibitors (Hartman *et al.*, 1994; Richard *et al.*, 1995; Zhu *et al.*, 1995).

Conflicting experimental results have been obtained with ·NO synthase inhibitors during ischaemia. In several reports, these agents have been shown to exacerbate the myocardial stunning and ischaemic damage to the heart (Hasebe *et al.*, 1993; Williams *et al.*, 1995). However, in other studies, inhibition of ·NO synthase during ischaemia was shown to be protective (Matheis *et al.*, 1992; Woolfson *et al.*, 1995). The protective action was abolished by pretreatment with an adenosine receptor antagonist, suggesting that inhibition of ·NO synthase in the heart triggered the compensatory release of adenosine, which exerted positive effects during ischaemia (Woolfson *et al.*, 1995). Thus, there is ample evidence in support of the cardioprotective role of ·NO released from the constitutive ·NO synthase. Recent studies have shown that various ·NO donors protect the heart against regional myocardial ischaemia *in vivo* and against global ischaemia during heart preservation (Pinsky *et al.*, 1994). Previously we have shown that postischaemic reperfusion of GSNO-treated and control hearts with constant flow did not abolish the protective action of GSNO (Konorev *et al.*, 1995b). Thus, the increased rate of coronary flow during reperfusion in GSNO-MEE-treated group is unlikely to play a major role in the improved recovery of contractile function.

Detection of nitrosylmyoglobin (MbNO) formation in the presence of GSNO-MEE

GSNO-MEE induced the formation of MbNO in the heart. Myoglobin which is abundant in muscle cells, has previously been used to trap ·NO in myocardial cells (Konorev *et al.*, 1996). The formation of the MGD-Fe^{2+}-NO adduct indicated ·NO trapping in the extracellular space. However, MbNO was detected even in the presence of the extracellular ·NO trap MGD-Fe^{2+} because myoglobin haeme iron has a higher affinity for ·NO (Jongeword *et al.*, 1988).

MbNO formed during ischaemia in the presence of GSNO-MEE persisted throughout the early reperfusion period with oxygen-saturated buffer. This implies a rather slow displacement of ·NO with oxygen, and the inability of myoglobin to facilitate the intracellular oxygen transport to mitochondria during early reperfusion. Inactivation of myoglobin has been reported to reduce the rate of mitochondrial respiration and to elevate the NADH/NAD^+ ratio in myocardial cells (Doeller & Wittenberg, 1991; White & Wittenberg, 1993).

Elevation of myocardial cyclic GMP by GSNO-MEE

GSNO-MEE caused a significant accumulation of cyclic GMP in the heart, during both ischaemia and aerobic perfusion. Several cell types in the heart, including smooth muscle cells and cardiac myocytes, possess guanylyl cyclase activity and could contribute to the elevation of cyclic GMP by GSNO-MEE. Based on the present data, it is difficult to pinpoint the exact cell type responsible for elevation of cyclic GMP. In control hearts a decline in myocardial cyclic GMP levels was observed during ischaemia. These data do not agree with those of Depre & Hue (1994), who reported an increase in cyclic GMP content during myocardial ischaemia. Different ischaemic conditions (cardioplegic ischaemia *vs.* ischaemia in KHB in their study) could contribute to this discrepancy. Pinsky *et al.* (1994) used the lactated Ringer solution for ischaemic heart preservation and found decreased levels of ·NO in myocardial tissue. However, myocardial cyclic GMP level was not measured in that study.

The supplementation of heart transplants with an analogue of cyclic GMP was shown to protect against ischaemia, possibly through vascular protection (Pinsky *et al.*, 1994). Besides its action on the vasculature and blood cell elements, cyclic GMP also affects the metabolic and ion fluxes in cardiac myocytes. It has been shown to inhibit L-type Ca^{2+} currents in different species (Tohse & Sperelakis, 1991; Mery *et al.*, 1991; Wahler & Dollinger, 1995) and to enhance the phosphorylation of phospholamban, increasing the rate of Ca^{2+} sequestration into the sarcoplasmic reticulum (Bartel *et al.*, 1995). Cyclic GMP antagonizes the activation of metabolism and Ca^{2+} fluxes induced by cyclic AMP-dependent hormones and transmitters via inhibition of phosphorylation of ion channels and contractile proteins (Hartzell & Fischmeister, 1986; Bartel *et al.*, 1993). However, Shah *et al.* (1994) reported that a membrane-permeable analogue of cyclic GMP induced an increase in free Ca^{2+} concentration in 50% of rat isolated cardiac myocyctes that was associated with a decreased sensitivity of myofibres to Ca^{2+}.

Beitner *et al.* (1977) showed that cyclic GMP, at concentration as low as 10 nmol l^{-1}, inhibited the key glycolytic enzyme, phosphofructokinase, isolated from rat heart tissue. It also antagonized the action of its allosteric activators, such as cyclic AMP, AMP, and fructose-1,6-diphosphate (Beitner *et al.*, 1977), which could result in the inhibition of glycolysis and, consequently, glycogenolysis. In addition, the cyclic GMP analogue and cyclic GMP-generating agents were shown to decrease NADH and lactate accumulation and to increase ATP and phosphocreatine contents in mildly hypoxic (20–50% O_2 saturation) rat atria (Laustiola *et al.*, 1983) and right ventricle (Ljusegren & Axelsson, 1993). However, in the present study, myocardial ATP and lactate levels were similar in GSNO-MEE and control hearts, suggesting similar rates of anaerobic glycolysis in both groups. These discrepancies could be explained by a more severe degree of ischaemia in our ex-

periments and by the direct effects of GSNO-MEE on myocardial metabolism, which could offset the cyclic GMP-mediated metabolic changes.

GSNO-MEE and myocardial metabolites

We report here a novel observation that ·NO donors reduce the glycogen content in aerobically-perfused hearts. The glycogen reduction was associated with the decrease in myocardial Glu-6-P levels. In several recent reports, it has been shown that ·NO inhibits mitochondrial respiration via a reversible inactivation of cytochrome oxidase (Schweizer & Richter, 1994; Cleeter *et al.*, 1994). Formation of MbNO can also contribute to the inhibition of mitochondrial respiration (Doeller & Wittenberg, 1991). Inhibition of cellular respiration has been shown to cause rapid activation of glycogen phosphorylase, which is converted to the active 'a' form during oxygen deficiency (Neely & Morgan, 1974) or inhibition of cytochrome oxidase by cyanide (Vandebroeck *et al.*, 1988).

Reduction in glycogen was also observed during the induction of cardioplegia, when myocytes appeared to be more sensitive to glycogenolytic effects of GSNO-MEE; a 4 min induction of cardioplegia caused glycogen depletion to the same extent as a 10 min perfusion with oxygenated KHB. This could be due to the absence of exogenous energetic substrate and lower O_2 tension in cardioplegic solution, which was not saturated with 95% O_2/5% CO_2 gas mixture. The cytochrome oxidase was shown to be more susceptible to inhibition by ·NO at lower oxygen tensions (Schweizer & Richter, 1994).

Therefore, GSNO-MEE-treated hearts went into ischaemia with a decreased amount of glycogen. Preischaemic glycogen reduction was shown to protect against ischaemia in rat isolated hearts (Neely & Grotyohann, 1984; Kupriyanov *et al.*, 1988). Protection of preconditioned myocardium was suggested to result from preischaemic glycogen reduction, and replenishment of myocardial glycogen stores abolished the myocardial protection caused by preconditioning (Wolfe *et al.*, 1993). However, it has not been shown, whether preischaemic glycogen reduction *per se* or associated attenuation of intracellular acidosis during ischaemia is responsible for the increased myocardial protection. In the present study, lactate accumulation was similar in control and GSNO-MEE-treated groups, which probably indicated similar rates of glycolytic ATP production and pH values during ischaemia (Gevers, 1977).

In our previous work, GSNO, in contrast to its ester analogue, GSNO-MEE, caused a significant reduction in myocardial lactate accumulation during cardioplegic ischaemia, but the glycogen content was not measured in that study (Konorev *et al.*, 1995b). We suggested that a reduction in glycolytic metabolites could contribute to myocardial protection. Based on the present study, it appears that the reduction in myocardial lactate during ischaemia is not the major determinant of protective action of nitrosothiols.

Conclusion

Inclusion of GSNO-MEE in the cardioplegic solution improved the functional recovery of the rat isolated heart subjected to ischaemia and reperfusion. GSNO-MEE released ·NO in myocardial cells as shown by intracellular MbNO formation and increased myocardial cyclic GMP. Elevated cyclic GMP could contribute to the protective action of GSNO-MEE because of its favourable effects on myocardial Ca^{2+} fluxes and reduced sensitivity of myofibres to Ca^{2+}. GSNO-MEE reduced the glycogen content during aerobic perfusion and indication of cardioplegia, but did not alter myocardial ATP and lactate levels during ischaemia as compared with control hearts. It is also possible that preischaemic glycogen reduction may confer increased protection on the heart during cardioplegic ischaemia.

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