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S-Nitrosothiol-induced rapid cytochrome *c* release, caspase activation and mitochondrial permeability transition in perfused heart

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Abstract

Nitric oxide (NO) is a physiological signalling molecule, however, at high concentrations NO is cytotoxic, and has been implicated in a wide range of inflammatory, ischaemic and degenerative diseases, including heart failure. We investigated whether NO or *S*-nitrosothiols can induce apoptosis in perfused heart, and whether it is mediated *via* the mitochondrial pathway of caspase activation. We found that perfusion of rat hearts with a physiological *S*-nitrosothiol, *S*-nitrosoglutathione, at 0.4–1 mM concentrations for just 10 min caused the release of cytochrome *c* from mitochondria into the cytosol, inhibition of mitochondrial respiration and caspase activation. Inhibited mitochondrial respiration was restored when exogenous cytochrome *c* was added to mitochondria, indicating that respiratory inhibition was caused by lack of cytochrome *c* in mitochondria. Release of cytochrome *c*, respiratory inhibition and caspase activation were prevented when hearts were pre-perfused with cyclosporin A, suggesting that mitochondrial permeability transition pore was involved. In contrast, perfusion of the hearts with diethylenetriamine/NO adduct releasing similar levels of NO to the *S*-nitrosoglutathione had no measurable effect on the heart. These data suggest that *S*-nitrosothiols are potent inducers of apoptosis in the heart and that *S*-nitrosothiol-induced apoptosis is mediated by mitochondrial permeability transition but not *via* NO.

Keywords: Nitric oxide; Perfused heart; Mitochondria; Respiration; Apoptosis

1. Introduction

NO has two general roles in the body: (i) at low levels NO from constitutive isoforms of NO synthase acts as a physiological signalling molecule, and (ii) at high levels NO from inducible nitric oxide synthase (iNOS) is a cytotoxic agent of the innate immune system [1]. Inflammatory stimuli, such as cytokines, bacteria or viruses, induce the expression of iNOS in a wide range of cells, including macrophages, endothelial cells and cardiomyocytes [2–4]. Many cardiovascular pathologies, such as sepsis, ischaemia and heart failure, also result in iNOS expression in the heart [5–8]. NO has a variety of actions within the heart, including at low concentrations vasodilation and increased contractility, while at high concentrations NO inhibits mitochondrial respiration and decreases contractility. Low levels of NO or iNOS expression can suppress heart cell death induced by ischaemia/reperfusion, and NO is thought to be one of the main mediators of ischaemic preconditioning [9,10]. However, NO has also been reported to induce necrosis or apoptosis in heart cells [11–13], but the mechanisms or reasons for apparently contradictory actions remain obscure.

NO can react, *via* intermediates, such as N_2O_3, with thiols (e.g. glutathione and protein thiols) to produce *S*-nitrosothiols, a process known as *S*-nitrosation (addition of an NO^+ group). The NO^+ group can be transferred directly between thiols, a process known as transnitrosation or transnitrosylation. Alternatively, NO may be released from

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Abbreviations: DETA/NO, diethylenetriamine/NO adduct; GSNO, *S*-nitrosoglutathione; iNOS, inducible nitric oxide synthase; NO, nitric oxide; MPT, mitochondrial permeability transition.

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S-nitrosothiols by Cu^{+} or light. A variety of proteins are thought to be regulated by *S*-nitrosation, for example, *S*-nitrosation of the ryanodine receptor in heart is thought to cause calcium release from intracellular stores [14]. High levels of *S*-nitrosothiols may be released from iNOS expressing cells [15,16].

In certain conditions *S*-nitrosothiols may cause activation of the mitochondrial permeability transition (MPT) in isolated mitochondria and consequent cytochrome *c* release [17]. MPT is a large increase in the permeability of the mitochondrial inner membrane to all small molecules, due to opening of the MPT pore, classically induced by high calcium and oxidants [18,19]. MPT results in uncoupling of oxidative phosphorylation, ATP hydrolysis, mitochondrial swelling and/or release of mitochondrial cytochrome *c*. MPT has been invoked as the mechanism by which heart ischaemia and/or reperfusion induces heart cell necrosis or apoptosis [19,20]. MPT may cause necrosis by depleting cellular ATP [21,22]. Alternatively, if ATP levels do not fall too low, MPT may induce apoptosis by causing mitochondrial swelling, rupture of the outer membrane and release of cytochrome *c*, which may then initiate the caspase cascade *via* activating the apoptosome [22,23].

In this work, we tested whether a physiological *S*-nitrosothiol, *S*-nitrosoglutathione (GSNO), or an NO donor, diethylenetriamine/NO adduct (DETA/NO), which is thought to release NO only, could induce cytochrome *c* release, mitochondrial dysfunction and caspase activation in perfused heart. We found that 1 mM GSNO, but not 1 mM DETA/NO, releasing a similar amount of NO, activated all these changes within 10 min of perfusion. Furthermore, GSNO-induced cytochrome *c* release, mitochondrial dysfunction and caspase activation were prevented by pre-perfusing the heart with 0.1 μM cyclosporin A, suggesting that all these changes were a consequence of GSNO-induced MPT.

2. Materials and methods

Hearts from male Wistar rats were used in the experiments. Hearts were perfused with a Langendorff perfusion system at a pressure of 40 cmH_2O with Krebs–Henseleit buffer (11 mM glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.7 mM $MgSO_4$ and 0.7 mM Na pyruvate, pH 7.2, at 37°, gassed with 95% O_2/5% CO_2) for 5 min to stabilise the hearts. Then, where indicated, 0.25, 0.4, 0.5 or 1 mM GSNO (Sigma) or 1 mM DETA/NO (Alexis) was added and perfusion was continued for another 10 min. In the experiments on the effect of cyclosporin A, 0.1 μM cyclosporin A (Calbiochem) was present already during the initial stabilisation period. Control hearts were perfused with a buffer only for 10 min (after 5 min of stabilisation). The flow rate of perfusate was 20–23 mL/min and was similar in all experimental groups.

Mitochondria were isolated by standard procedures as described in [24]. Heart after excision was washed in cold 0.9% KCl solution and cut into small pieces. The tissue was homogenised in the medium (10 mL/g tissue) containing 180 mM KCl, 10 mM Tris–HCl, 2 mM EGTA, pH 7.7 (4°), for 25–45 s. The homogenate was centrifuged 5 min at 750 *g* and after that 10 min at 6750 *g*. The mitochondrial pellet was resuspended in 0.15–0.25 mL buffer containing 180 mM KCl, 20 mM Tris–HCl, 1 mM EGTA, pH 7.3. The supernatant was additionally centrifuged for 30 min at 10,000 *g* to obtain the cytosolic fraction. Mitochondrial and cytosolic protein concentrations were determined by the biuret method.

Steady-state levels of NO released from GSNO and DETA/NO in the buffer collected during and at the end of heart perfusions with NO donors were measured using an NO electrode (World Precision Instruments) and were calibrated with NO-saturated water (2 mM).

Mitochondrial respiration was measured with a Clarke-type oxygen electrode in 1 mL of incubation medium containing 110 mM KCl, 10 mM Tris–HCl, 5 mM KH_2PO_4, 50 mM creatine, 1 mM $MgCl_2$, and 1 mM pyruvate + 1 mM malate, pH 7.2. Mitochondrial state 3 respiration rate was obtained by adding 1 mM ATP, which was converted to ADP by creatine kinase (4 IU/mL). In some experiments mitochondrial respiration was measured in the presence of 30 μM exogenous cytochrome *c*.

To measure the content of cytochrome *c* and cytochrome *a* mitochondria were solubilised with 1% Triton X-100. Sodium hydrosulphite-reduced minus hydrogen peroxide-oxidised absorption difference spectra were recorded with a Hitachi-557 spectrophotometer, and content of mitochondrial cytochromes was calculated using the absorption difference at the wavelength pair 550/535 nm, ε= 18.5 mM/cm for cytochrome *c* and 650/500 nm, ε= 16.5 mM/cm for cytochrome *a* [25].

Cytochrome *c* in cytosolic fractions of the hearts were identified by the quantitative sandwich enzyme immunoassay using Quantikine Rat/Mouse Cytochrome *c* determination kit (R&D Systems). Cytosolic fraction protein was dissolved in 0.5% Triton X-100 and further procedures were performed according to manufacturer's protocol.

Activity of caspases was measured in cytosolic fractions isolated from heart homogenates. 1 mg/mL of total cytosolic protein was incubated for 1 hr in buffer containing 10% sucrose, 50 mM HEPES, 1 mM $MgCl_2$, 1 mM ATP (pH 7.4, 37°) and 0.1 mM z-DEVD-*p*-nitroanilide, a caspase substrate. The hydrolysis of caspase substrate was followed spectrophotometrically at 405 nm and was calibrated with *p*-nitroanilide. DEVD-cleaving activity was completely suppressed by 20 μM DEVD-CHO, a reversible inhibitor of caspase-3.

Data are expressed as means ± SE of at least three separate experiments. Statistical comparison between experimental groups was performed using Student's *t*-test. Statistical significance was assumed at $P < 0.05$.

3. Results

To test whether NO can induce apoptosis in the heart by the mitochondrial route, we perfused hearts with two different NO donors, GSNO or DETA/NO, and then measured the mitochondrial and cytosolic contents of cytochrome *c*. GSNO in a concentration-dependent manner induced release of cytochrome *c* from mitochondria: a 20–60% decrease in mitochondrial cytochrome *c* content was observed in hearts perfused with 0.4–1 mM GSNO (Fig. 1A). In contrast, DETA/NO had no effect on mitochondrial cytochrome *c* level even at 1 mM concentration. Both donors, GSNO or DETA/NO, had no significant effect on cytochrome *a* level in mitochondria: 0.358 ± 0.046 nmol/mg protein in control compared to 0.317 ± 0.025 nmol/mg protein in hearts perfused with 1 mM GSNO or 0.330 ± 0.020 nmol/mg protein perfused with 1 mM DETA/NO. Note, that the steady-state level of NO released from 1 mM GSNO or 1 mM DETA/NO in the perfusate were similar and in the range of 0.88–1.20 μM measured by NO electrode. Cytochrome *c* released from mitochondria accumulated in the cytosols as can be seen in Fig. 1B. These data suggest that *S*-nitrosothiols but not DETA/NO (which is thought to release NO only) can induce rapid

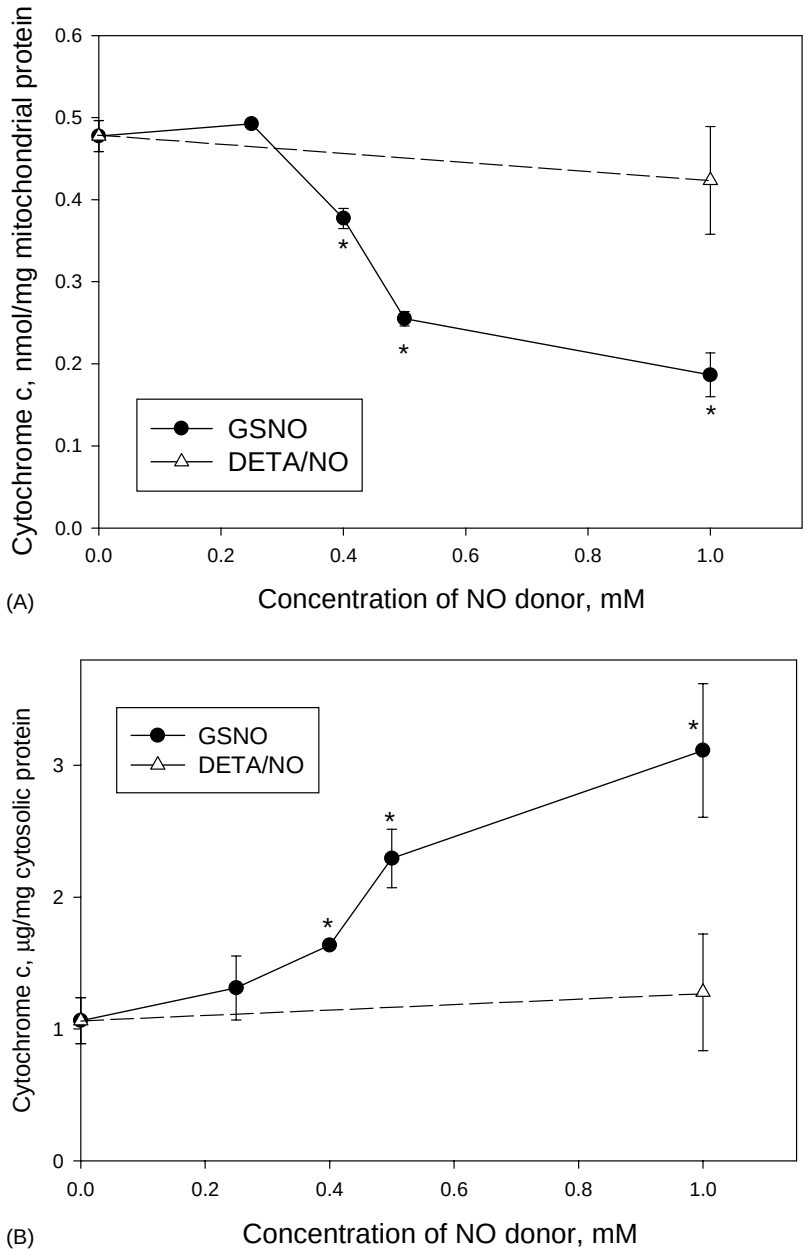

Fig. 1. Concentration-dependent effects of NO donors on cytochrome *c* content in mitochondria (A) and in cytosolic fractions (B). The hearts were perfused for 10 min with GSNO or DETA/NO at the indicated concentrations. Cytochrome *c* content was measured in subsequently isolated mitochondria (A) by spectrophotometric method or in cytosolic extracts (B) using solid phase ELISA. ∗—statistically significant ($P < 0.05$) effect of GSNO on cytochrome *c* level compared to control level (at zero concentration of GSNO).

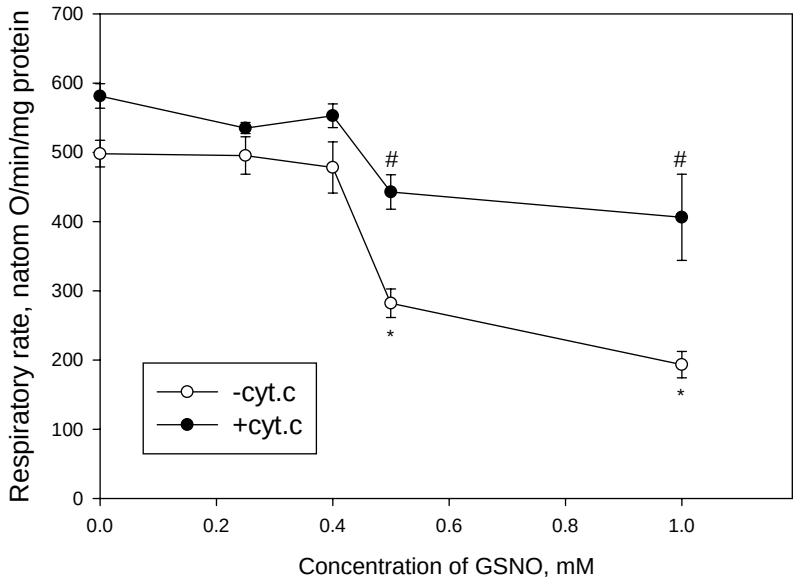

Fig. 2. Effect of exogenous cytochrome *c* on GSNO-inhibited mitochondrial respiration rate. Mitochondria were isolated from hearts perfused with the indicated concentrations of GSNO or with buffer only (control). Mitochondrial respiration was measured using 1 mM pyruvate + 1 mM malate as respiratory substrates. Where indicated, 30 μM cytochrome *c* was added into the mitochondrial incubation medium. ∗—statistically significant ($P < 0.01$) effect of GSNO compared to control (at zero concentration of GSNO); #—statistically significant ($P < 0.01$) effect of exogenous cytochrome *c* compared to mitochondrial respiration in the absence of cytochrome *c* at the same concentration of GSNO.

release of cytochrome *c* from mitochondria in perfused heart.

Loss of cytochrome *c* from mitochondria caused impairment of mitochondrial respiratory function (Fig. 2): state 3 respiration with pyruvate as substrate was decreased by 43 and 57% in mitochondria isolated from hearts perfused with 0.5 and 1 mM GSNO, respectively, compared to control (hearts perfused with buffer only). Re-addition of cytochrome *c* to these mitochondria restored the respiratory rate almost to the control level. As exogenous cytochrome *c* can stimulate respiration only if there is damage to mitochondrial outer membrane and deficiency of endogenous cytochrome *c*, these findings suggest that GSNO-induced inhibition of mitochondrial respiration was due to

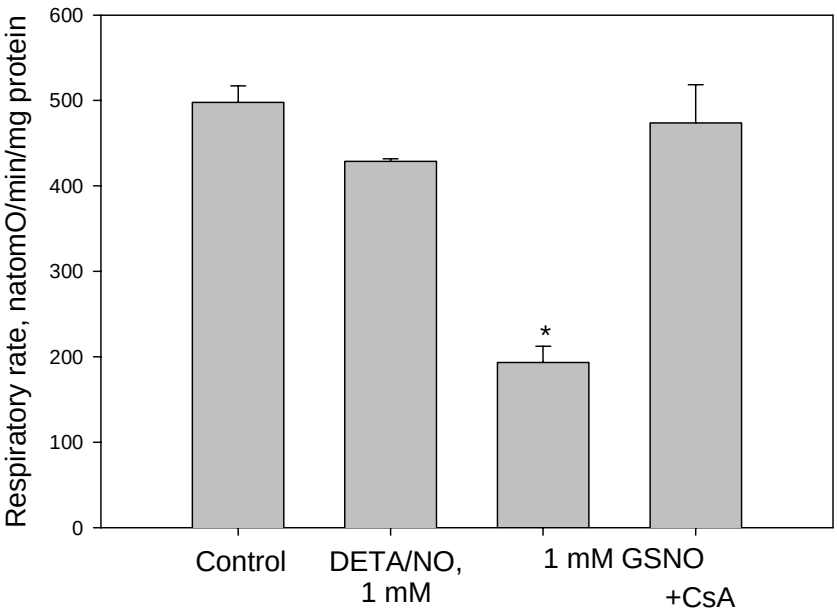

Fig. 3. Effect of cyclosporin A on GSNO-inhibited mitochondrial respiratory rate. Mitochondria were isolated from hearts perfused with 1 mM DETA/NO, 1 mM GSNO, 0.1 μM cyclosporin A (CsA) + 1 mM GSNO or with buffer only (control). Mitochondrial respiration was measured using 1 mM pyruvate + 1 mM malate as substrates. Perfusion with cyclosporin A alone had no effect on mitochondrial respiration. ∗—statistically significant ($P < 0.01$) effect compared to control.

the loss of cytochrome *c*. Perfusion with lower concentrations of GSNO (0.25 and 0.4 mM) had no significant effect on the respiration of subsequently isolated mitochondria, and the effect of added cytochrome *c* was the same as in control mitochondria (8–15% stimulation of respiration), though there was a small (20%) decrease in mitochondrial cytochrome *c* content after perfusion with 0.4 mM GSNO. Perfusion of the hearts with 1 mM DETA/NO had no detectable effect on mitochondrial respiration (Fig. 3).

Next, we investigated whether GSNO-induced cytochrome *c* release and mitochondrial dysfunction is related to MPT. For that hearts were pre-perfused with 0.1 μM cyclosporin A, a specific inhibitor of MPT, and then hearts were perfused for 10 min with 1 mM GSNO. As can be seen in Figs. 3 and 4, cyclosporin A completely prevented GSNO-induced inhibition of state 3 respiration rate as well as the release of cytochrome *c* from mitochondria and its accumulation in the cytosols. This suggests that perfusion of the hearts with GSNO induces opening of MPT pore which causes cytochrome *c* release and subsequent inhibition of mitochondrial respiration.

Apoptosis is executed by specific proteases: the caspases. Therefore, we measured activity of caspases in cytosolic extracts from hearts perfused with NO donors

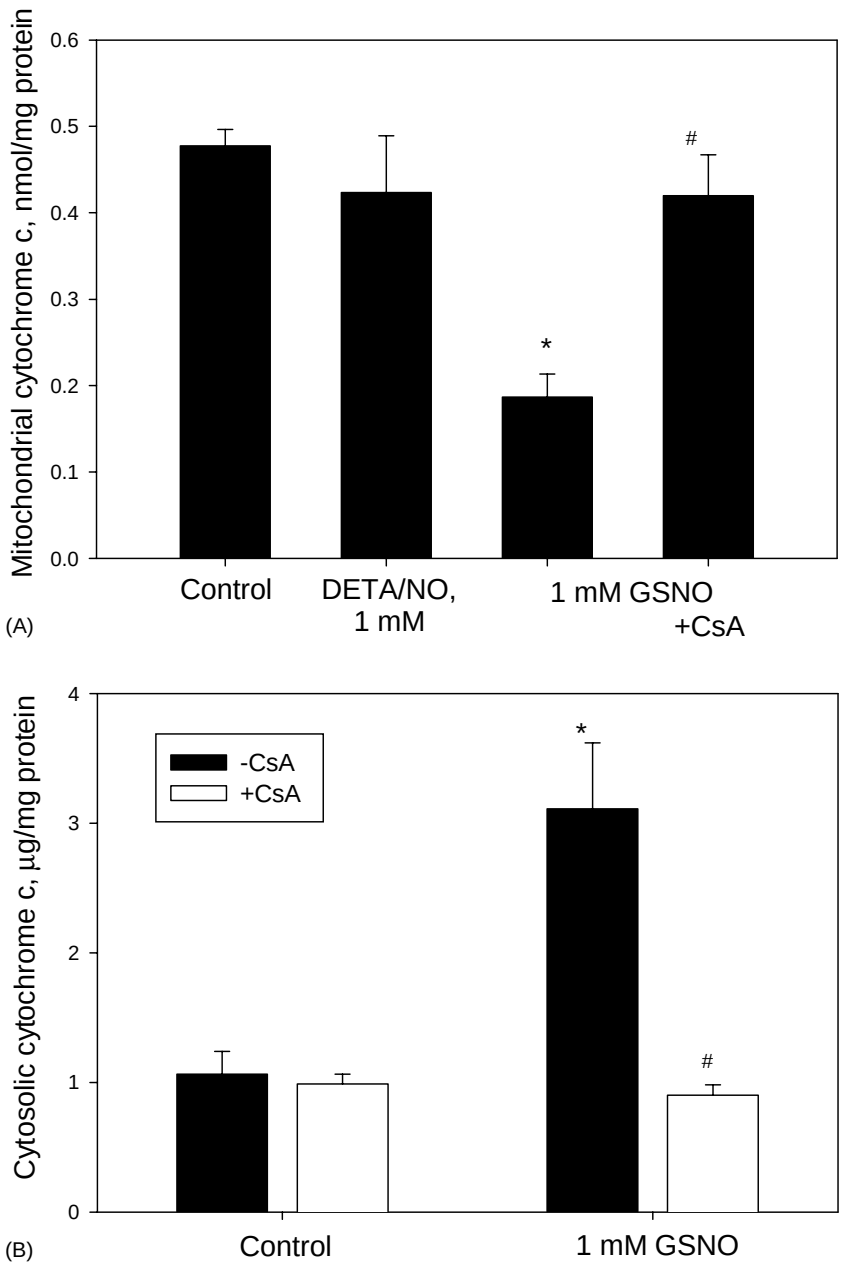

Fig. 4. Effect of cyclosporin A on GSNO-induced cytochrome *c* release from mitochondria into cytosol. (A) Mitochondrial content of cytochrome *c*; (B) content of cytochrome *c* in cytosolic extracts. Content of cytochrome *c* was measured in the same preparations of isolated mitochondria as in Fig. 3. ∗—statistically significant ($P < 0.01$) effect compared to control; #—statistically significant ($P < 0.01$) effect of cyclosporin A (CsA) if compared to hearts perfused with 1 mM GSNO.

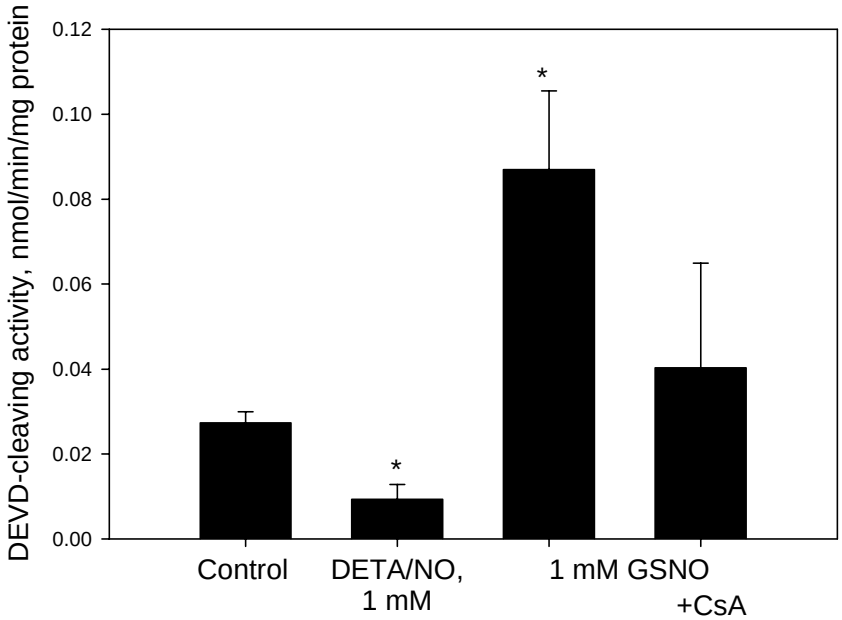

Fig. 5. Effect of cyclosporin A on GSNO-induced activation of caspases. DEVD-cleaving activity was measured in cytosolic extracts from hearts perfused with 1 mM DETA/NO, 1 mM GSNO, 0.1 μM cyclosporin A (CsA) + 1 mM GSNO or with buffer only (control). ∗—statistically significant ($P < 0.01$) effect compared to control.

as an indicator of apoptotic cell death. Figure 5 shows that in hearts perfused with DETA/NO there was no activation of caspases, moreover, the basal level of DEVD-cleaving activity was reduced by ∼60%. In contrast, perfusion of hearts with 1 mM GSNO caused activation of caspases: DEVD-cleaving activity increased by ∼220% compared to the control level. GSNO-induced caspase activity was significantly reduced (close to the control level) in hearts loaded with cyclosporin A, indicating that *S*-nitrosothiols-induced apoptosis may be mediated by MPT.

4. Discussion

1 mM GSNO induced cytochrome *c* release, mitochondrial dysfunction and caspase activation within 10 min of perfusion. This is a very rapid induction of cell death. However, 1 mM DETA/NO, releasing a similar level of free NO, caused none of these changes, suggesting that the GSNO-induced changes are not mediated by free NO, and thus are probably mediated by transnitrosation reactions. NO itself may be relatively ineffective in the heart due to its rapid reaction with myoglobin. The failure of NO to induce an acute cell death programme does not, however, exclude the possibility that longer term exposure to NO may induce cell death. On the other hand, DETA/NO did lower the basal caspase activity significantly, suggesting that NO may acutely inhibit caspase activity.

GSNO-induced cytochrome *c* release, mitochondrial dysfunction and caspase activation were prevented by pre-perfusing the heart with 0.1 μM cyclosporin A. Cyclosporin A is an inhibitor of MPT, and 0.1 μM cyclosporin A is known to inhibit MPT relatively specifically in heart [26]. Cyclosporin A can inhibit other proteins, such as calcineurin, but at higher concentrations [27]. Thus, the ability of 0.1 μM cyclosporin A to block GSNO-induced cytochrome *c* release, mitochondrial dysfunction and caspase activation implies that MPT mediates these changes. We have previously shown that GSNO and other *S*-nitrosothiols can induce MPT and cytochrome *c* release from isolated heart mitochondria, whereas authentic NO or NO from DETA/NO was unable to do so [17], and that addition of cytochrome *c* (plus dATP) to heart cytosol is sufficient to cause caspase activation [28]. We have also previously shown that *S*-nitrosothiols can induce caspase activation in macrophages by inducing MPT [17]. Thus, in the present work, GSNO appears to activate caspases in the heart by entering heart cells, and causing MPT, resulting in cytochrome *c* release and consequently caspase activation. However, GSNO might alternatively or additionally induce MPT in heart by causing calcium release from sarcoplasmic reticulum or inducing oxidative stress, as *S*-nitrosothiols are known to activate the ryanodine receptor in heart [14], and can deplete cellular glutathione [29].

The inhibition of mitochondrial respiration in GSNO perfused hearts appears to be due to MPT-induced cytochrome *c* release, as it was prevented by cyclosporin A and reversed by exogenous cytochrome *c*. Such inhibition of mitochondrial respiration might cause contractile dysfunction and/or necrosis. GSNO-induced MPT may itself cause contractile dysfunction and/or necrosis by causing ATP depletion. In principle, the GSNO-induced MPT might have occurred during the mitochondrial isolation rather than the heart perfusion, but we think this unlikely because

the isolation medium (as opposed to the perfusion medium) is designed to prevent MPT (high EGTA, low oxygen, low temperature) and will dilute out GSNO.

The levels of GSNO used in this study are much higher than the physiological levels of *S*-nitrosothiols measured in healthy plasma (30–120 nM) [16,30,31]. However, *S*-nitrosothiol levels rise to micromolar concentrations during inflammation/endotoxemia due to iNOS expression [16,30–32]. Also the cell death induced by GSNO was very rapid, and lower levels might induce death more slowly, as they do in other cell types [32]. iNOS is expressed in the heart in sepsis, bacterial or viral infection, ischaemia and heart failure [5–8]. It is therefore possible but speculative that part of the pathology of these conditions is mediated by *S*-nitrosothiols activating MPT, cytochrome *c* release, mitochondrial dysfunction and caspase activation.

Acknowledgments

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