Glycine protects cardiomyocytes against lethal reoxygenation injury by inhibiting mitochondrial permeability transition

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Post-ischaemic reperfusion may precipitate cardiomyocyte death upon correction of intracellular acidosis due in part to mitochondrial permeability transition. We investigated whether glycine, an amino acid with poorly understood cytoprotective properties, may interfere with this mechanism. In cardiomyocyte cultures, addition of glycine during re-energization following 1 h of simulated ischaemia (NaCN/2-deoxyglucose, pH 6.4) completely prevented necrotic cell death associated with pH normalization. Glycine also protected against cell death associated with pH normalization in reoxygenated rat hearts. Glycine prevented cyclosporin-sensitive swelling and calcein release associated with re-energization in rat heart mitochondria submitted to simulated ischaemia or to Ca^{2+} stress under normoxia. NMR spectroscopy revealed a marked glycine depletion in re-energized cardiomyocytes that was reversed by exposure to 3 mM glycine. These results suggest that intracellular glycine exerts a previously unrecognized inhibition on mitochondrial permeability transition in cardiac myocytes, and that intracellular glycine depletion during myocardial hypoxia/reoxygenation makes the cell more vulnerable to necrotic death.

Mitochondria are increasingly recognized as central regulators of cell death and survival in cardiac myocytes exposed to ischaemia–reperfusion (Duchen, 1999). A prominent player in the mitochondrial death pathway is the mitochondrial permeability transition pore, a largely unselective megachannel connecting the mitochondrial matrix with the cytosol and allowing the passage of molecules up to 1.5 kDa. During reperfusion, mitochondrial permeability transition (MPT) may be triggered by elevated Ca^{2+} concentrations, radical oxygen species and normalization of intracellular pH, and it is followed by the collapse of mitochondrial membrane potential, interruption of ATP synthesis, release of pro-apoptotic molecules into the cytosol (Petronilli *et al.* 2001; Di Lisa *et al.* 2001) and profound ionic disturbances that may result in necrotic or apoptotic death (Lemasters *et al.* 1998; Kim *et al.* 2003). It has been recently proposed that inhibition of MPT may have a strong protective effect

by preventing the development of structural cell fragility (Hausenloy *et al.* 2003; Javadov *et al.* 2003).

The amino acid glycine (Gly) has been shown to exert a protective effect against cell death after diverse insults including ischaemia–reperfusion injury in different cell types other than cardiomyocytes, but the mechanism of this effect remains elusive (Nishimura *et al.* 1998; Schemmer *et al.* 1999; Nishimura & Lemasters, 2001; Dong *et al.* 2001; Zhang *et al.* 2003). In this study we investigated the effect of Gly on MPT and reoxygenation-induced cell death in cultured HL-1 cardiac myocytes, intact rat myocardium and rat heart mitochondria. The results show that Gly, an endogenous amino acid, may play a cardioprotective effect in reoxygenated cardiomyocytes by preventing MPT associated with pH normalization.

Methods

This study was performed on cultured HL-1 cardiac myocytes, Langendorf-perfused hearts from Sprague-Dawley rats, and isolated mitochondria. The experimental

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procedures conformed with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication no. 85-23, revised 1996), and were approved by the Research Commission on Ethics of the Hospital Vall d'Hebron.

Studies in HL-1 cardiomyocytes

HL-1 cardiac myocytes were plated at $20\,000$ cells cm^{-2} density in glass-bottomed culture dishes until a 70–80% confluence was achieved, as previously described (Ruiz-Meana *et al.* 2003).

Simulated ischaemia–reoxygenation. Cell pellets were submitted to 1 h simulated ischaemia (SI) by suspending them in a buffer containing (mM): NaCl 140, KCl 3.6, CaCl$_2$ 2, MgSO$_4$ 1.2, Hepes 20, NaCN 2, 2-deoxyglucose 20, at pH 6.4, 37°C. Reoxygenation was performed by centrifugation and resuspension of the pellet in a control normoxic buffer at pH 7.4, with 5 mM glucose.

Experimental interventions. The effect of modifying the reoxygenation buffer by lowering its pH (up to 6.4) or by adding Gly (3 mM) was investigated. The role of Ca^{2+} was studied by sequestration of either intra- or extracellular Ca^{2+} (addition of 30 μM BAPTA or removal of CaCl$_2$ in the presence of 2 mM EGTA).

Cell volume and viability. Changes in cell volume were determined by analysis of forward scatter (FACscalibur flow cytometer, Becton Dickinson, USA) and cell viability by exclusion of propidium iodide (PI). Double staining with annexinV–fluorescein isothiocyanate (FITC)–PI was used to quantify the contribution of apoptosis (annexin–FITC$^+$–PI$^-$ cells) to cell death.

Intracellular pH and Na$^+$. Changes in cytosolic [H$^+$] and [Na$^+$] during SI–reoxygenation were monitored by ratio-fluorescence imaging in HL-1 cells loaded, respectively, with 3 μM BCECF-AM or 5 μM SBFI (Molecular Probes, USA) (Ruiz-Meana *et al.* 2003).

Studies in isolated rat hearts

Heart preparation. After an intraperitoneal injection of sodium pentobarbital (100 mg kg^{-1}), the hearts from 18 Sprague-Dawley rats (280–350 g) were rapidly removed and perfused with an oxygenated Krebs solution at 37°C, as previously described (Rodriguez-Sinovas *et al.* 2003).

Experimental protocol. After 40 min of equilibration, hearts were submitted to 2 h of hypoxia at pH 6.4 followed by 1 h of reoxygenation. For hypoxic perfusion, a modified Krebs buffer (mM: NaCl 139.5, KCl 4.7, MgSO$_4$ 1.2, CaCl$_2$ 2.5, NaHCO$_3$ 3.5, KH$_2$PO$_4$ 1.2, and sucrose 11) was bubbled with 95% N$_2$–5% CO$_2$. Reoxygenation was performed by switching the hypoxic Krebs solution to a glucose-containing oxygenated (95% O$_2$–5% CO$_2$) Krebs solution. Hearts were allocated to one of three groups: (1) control reoxygenation ($n = 6$) at pH 7.4; (2) acidic reoxygenation ($n = 5$) at pH 6.4; (3) reoxygenation in the presence of 10 mM Gly ($n = 5$). Two additional hearts were used to calculate cumulative concentration–response curves of developed tension and transmembrane action potential to Gly (from 10^{-4} to 10^{-1} M, each concentration given during 15 min) under normoxia. Hearts were paced from the base (2.5 ms, 4 V pulses at 2.5 Hz) and transmembrane action potentials were recorded from the apex, as described (Rodriguez-Sinovas *et al.* 2003).

Enzyme release. Lactate dehydrogenase (LDH) release during reoxygenation was measured in samples taken from the effluent as previously described (Rodriguez-Sinovas *et al.* 2003).

Studies in isolated mitochondria

Isolation of mitochondria. Rat heart mitochondria were isolated by differential centrifugation, according to the method described by Holmuhamedov *et al.* (1998). Protein was adjusted to 1 mg ml^{-1}.

Experimental interventions. Mitochondria were submitted to either (1) simulated ischaemia (SI)– reoxygenation or (2) 15 min Ca^{2+} overload. To simulate ischaemia, the mitochondrial pellet was suspended in a metabolic inhibition buffer (mM: KCl 150, NaCl 7, Hepes 6, KH$_2$PO$_4$ 0.5, sucrose 50, MgCl$_2$ 1, ADP 0.1, CaCl$_2$ 0.01 and NaCN 2), at pH 6.4, for 1 h at 37°C. Reoxygenation was performed by switching to a NaCN-free solution containing (mM): KCl 150, NaCl 7, Hepes 6, KH$_2$PO$_4$ 0.5, sucrose 50, MgCl$_2$ 1, ADP 0.1, CaCl$_2$ 0.05, ATP 0.3, succinate 0.3, ascorbic acid 2.5, at pH 7.2. The effects of acid pH (6.4), Gly (3 mM) or cyclosporin A (CsA, 1 μM), as well as substrates (5 mM succinate, 5 mM pyruvate or 5 mM L-alanine) on MPT were assessed during the first 30 min of reoxygenation. Also, the contribution of Ca^{2+} to the MPT was tested by adding 2 mM EGTA or by increasing the extracellular Ca^{2+} to 200 μM in the reoxygenation buffer.

In the control group, mitochondria were submitted to the same experimental manipulations in a NaCN-free, low Ca^{2+} (0.1 μM) buffer containing (mM): KCl 150, NaCl 7, Hepes 6, KH_2PO_4 0.5, sucrose 50, $MgCl_2$ 1, ADP 0.1, ATP 0.3, succinate 5, ascorbic acid 2.5, at pH 7.2. The inhibitory effect of acid pH and Gly on MPT was also assessed in normoxic mitochondria submitted to 200 μM $CaCl_2$ for 15 min.

Swelling assay. Changes in mitochondrial volume were assayed spectrophotometrically in mitochondrial suspensions at 25°C using either 96-well-plates (Garlid *et al.* 1996) or 500 μl cuvettes. Light absorbance was determined at 520 nm under basal conditions, after 60 min of SI, and at the end of reoxygenation. Changes in light absorbance were normalized with respect to basal values. In normoxic Ca^{2+}-stressed mitochondria light absorbance was monitored throughout time following addition of 200 μM Ca^{2+} to the mitochondrial suspension. MPT was identified as a CsA-sensitive abrupt decrease in light absorbance, reflecting passive matrix swelling.

Assessment of calcein release. Calcein release was monitored in mitochondria using a 96-well fluoro-metry assay. The mitochondrial pellet was loaded with 1 μM calcein-AM at 37°C (Molecular Probes, USA), washed and submitted to 200 μM $CaCl_2$. At the end of the experiment, mitochondria were centrifuged and calcein fluorescence was determined (Excitation Wavelength: 485 nm, Emission Wavelength: 538 nm) in the pellet (intramitochondrial compartment) and in the supernatant (extramitochondrial compartment). MPT was identified as a CsA-sensitive loss of calcein from the intramitochondrial compartment. This effect was invariably associated with an increase in the calcein at the extramitochondrial compartment.

NMR spectroscopy

Cell pellets ($(3–5) \times 10^6$ cells per sample) were extracted with 1.2 ml of chloroform–methanol (1 : 2) at room temperature for 1 h. After addition of 0.5 ml chloroform and 0.5 ml of water, two phases were separated by centrifugation (1500 g, 5 min). The upper phase (methanol–water) was separated and dried under N_2. Dried extracts were dissolved with 400 μl of D_2O containing 1 mM 3-(trimethylsilyl) propionic acid (TSP) as chemical shift and concentration reference. Spectroscopy was performed at 27° C with a 400 MHz Bruker ARX spectrometer. Peak areas were measured by integration in fully relaxed spectra.

Statistical analysis

Statistical analysis was performed using commercially available software (SPSS for Windows 8.0). Comparisons between two independent groups were performed by Student's *t* test. The effect of SI–reoxygenation with or without Gly supplementation on intracellular Gly content was assessed by means of a *t* test for paired samples. Evaluation of unhomogeneity between multiple groups was performed by analysis of variance (ANOVA). Curves of LDH release were compared by repeated measures ANOVA. Data are expressed as the mean ± S.E.M. The significance level was set at 0.05.

Results

Cell death during SI–reoxygenation

SI did not result in detectable cell death (Fig. 1*A*). However, reoxygenation after 1 h of SI was associated with a marked increase in the number of PI^+ cardiomyocytes, reflecting sarcolemmal disruption (Fig. 1*A*). Sarcolemmal rupture occurred early upon reoxygenation, was fully observable at 30 min, and did not further increase when the duration of reoxygenation was prolonged to 2 h (data not shown). Apoptosis seemed not to contribute to cell death: the proportion of annexin$^+$–PI$^-$ cells after 30 min of reoxygenation was low and remained unchanged after 2 h of reoxygenation (data not shown). Furthermore, this proportion was not significantly modified by either SI or reoxygenation (Fig. 1*B*).

Cell death associated with reoxygenation was closely related to pH normalization and could be abolished when pH remained at 6.4 during reoxygenation (Fig. 1*A*). Addition of 3 mM Gly to the reoxygenation buffer completely prevented pH-dependent cell death (Fig. 1*A*). Chelation of Ca^{2+} from the extracellular medium with EGTA had no effect on reoxygenation-induced cell death (15.1 ± 0.4% and 16.3 ± 0.26% in control and EGTA-treated cells, respectively, n.s.). Similarly, intracellular Ca^{2+} sequestration with BAPTA, did not reduce cell death during reoxygenation (Fig. 1*C*).

Changes in cell volume, intracellular pH and Na$^+$ during SI–reoxygenation

SI induced a significant volume increase (cell swelling), followed by cell size correction upon restoration of energy and normalization of pH. Interestingly, the protective effect of acid pH and Gly on cell death was closely associated with an impaired capacity of cells to recover their volume (Fig. 2*A*). In both interventions,

dose–effect studies demonstrated a narrow inverse relationship between the degree of protection against reoxygenation-induced cell death and the magnitude of correction of cell volume (Fig. 2*B* and *C*).

Intracellular pH decreased rapidly during SI, reaching 6.4 ± 0.04 within 15 min, and recovered to 7.2 in the first 10 min of reoxygenation. Addition of Gly to the reoxygenation buffer did not produce any delay in

pH recovery (Fig. 3). Intracellular Na^+ concentration steadily rose during the whole SI period (from 1 ± 0 to 1.35 ± 0.05 a.u. of SBFI ratio fluorescence), remained stable during the 30 min reoxygenation (1.33 ± 0.1 a.u),
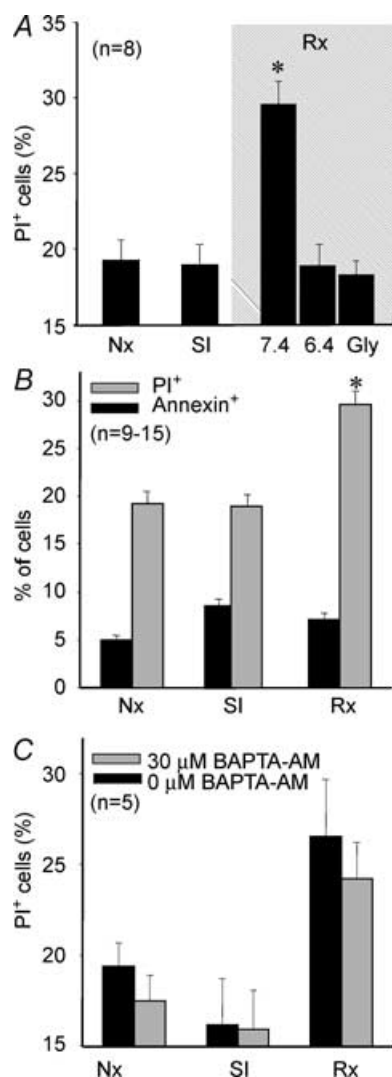

Figure 1. Cell death in cardiomyocytes submitted to 1 h SI–30 min reoxygenation
A, necrotic cell death (PI$^+$) when reoxygenation was performed at pH 7.4, at pH 6.4 or at pH 7.4 in the presence of 3 mm Gly. Cell death was associated with normalization of pH upon reoxygenation. Addition of Gly to the reoxygenation buffer prevented cell death associated with pH normalization. *B*, contribution of apoptosis (annexin$^+$–PI$^-$ cells) to total cell death was small and was not significantly increased by either SI or reoxygenation. *C*, sequestration of intracellular Ca^{2+} with BAPTA throughout the reoxygenation period did not afford any significant protection against cell death. $P < 0.05$ *versus* normoxic cells.
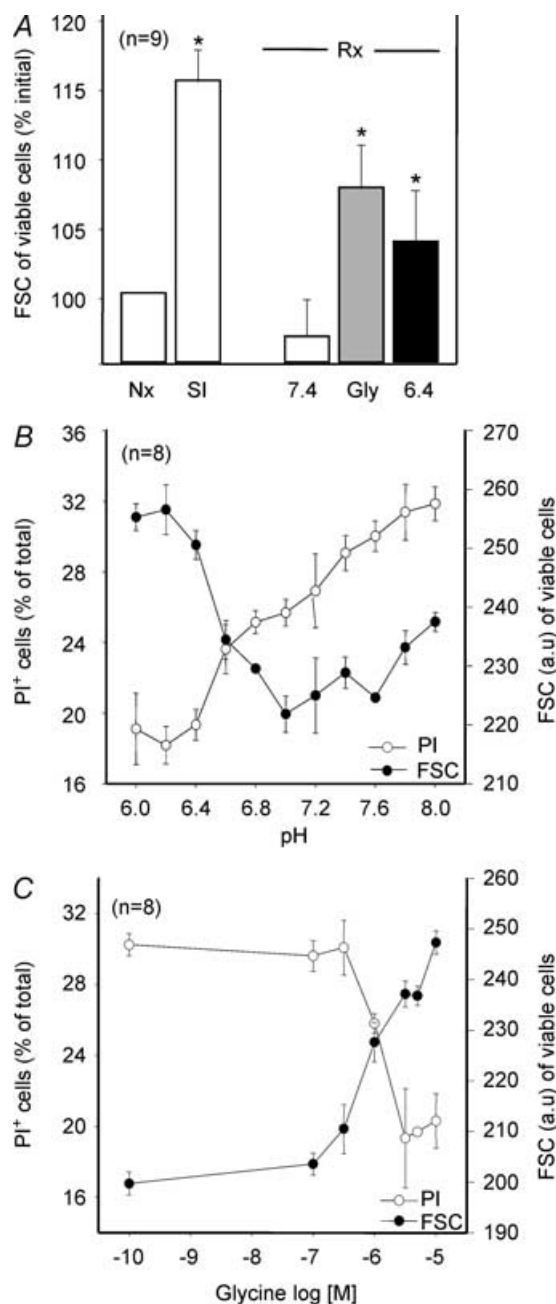
Figure 2. Effect of SI and reoxygenation on cardiomyocyte volume and death (PI$^+$ cells) under different conditions
A, cells submitted to SI experienced a significant increase in their volume, which was corrected upon reoxygenation at pH 7.4. Cell volume did not recover when Gly was present during reoxygenation or when reoxygenation was performed at pH 6.4. $P < 0.05$ *versus* normoxic cells. *B*, the degree of protection against death afforded by low pH closely correlated with the magnitude of the effect on cell volume. *C*, the degree of protection afforded by Gly was also closely correlated with its effect on cell volume.

and was not influenced by the presence of Gly in the reoxygenation buffer (1.32 ± 0.16 a.u., n.s.).

Effect of Gly on isolated rat hearts

Reoxygenation induced an important LDH release (Fig. 4*A*). In hearts submitted to reoxygenation at pH 6.4, total LDH release was reduced by approximately 50% (33.1 ± 6.0 U (g dry tissue)$^{-1}$ (60 min)$^{-1}$). Addition of 10 mM Gly during reoxygenation had a similar effect (LDH release was 37.4 ± 7.8 U (g dry tissue)$^{-1}$ (60 min)$^{-1}$, Fig. 4*B*). Normoxic perfusion with a Gly-containing Krebs solution, from 10^{-4} to 10^{-1} M, did not induce any change in transmembrane action potential characteristics,

conduction velocity or contractile function (data not shown).

Mitochondrial swelling during reoxygenation

In isolated mitochondria, reoxygenation at pH 7.2 after 1 h SI at pH 6.4 was associated with an increase in mitochondrial volume, as measured by a fall in light absorbance. Reoxygenation at pH 6.4 abolished mitochondrial swelling. Reoxygenation-induced swelling can be attributed to MPT since it was blocked by 1 μM CsA and could be precipitated by increasing Ca^{2+} to 200 μM during reoxygenation (Fig. 5*A*). MPT was not dependent on substrate availability, since the increase of succinate concentration from 0.3 to 5 mM, or addition of 5 mM pyruvate did not reduce mitochondrial swelling (Fig. 5*A*). Addition of 3 mM Gly to the reoxygenation buffer completely prevented mitochondrial swelling despite pH normalization (Fig. 5*B*). This protective effect against MPT could not be mimicked by 5 mM L-alanine
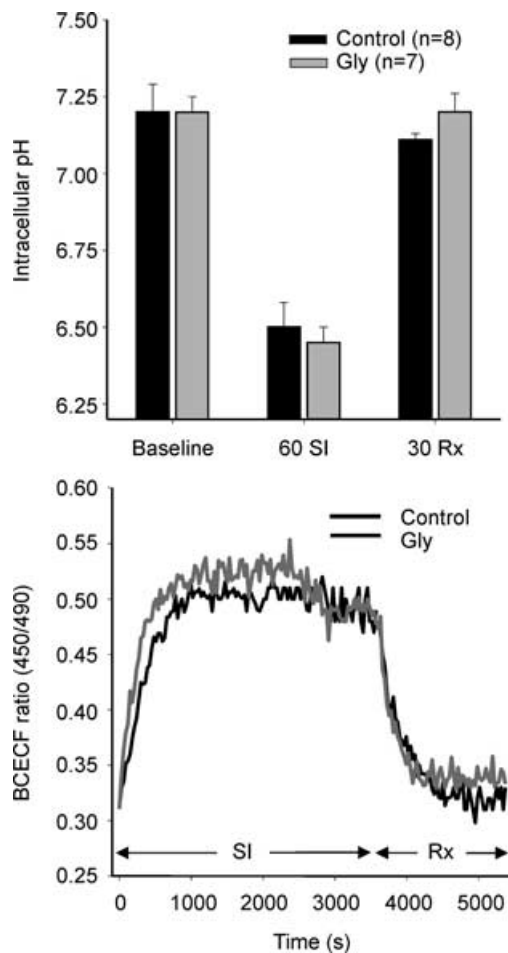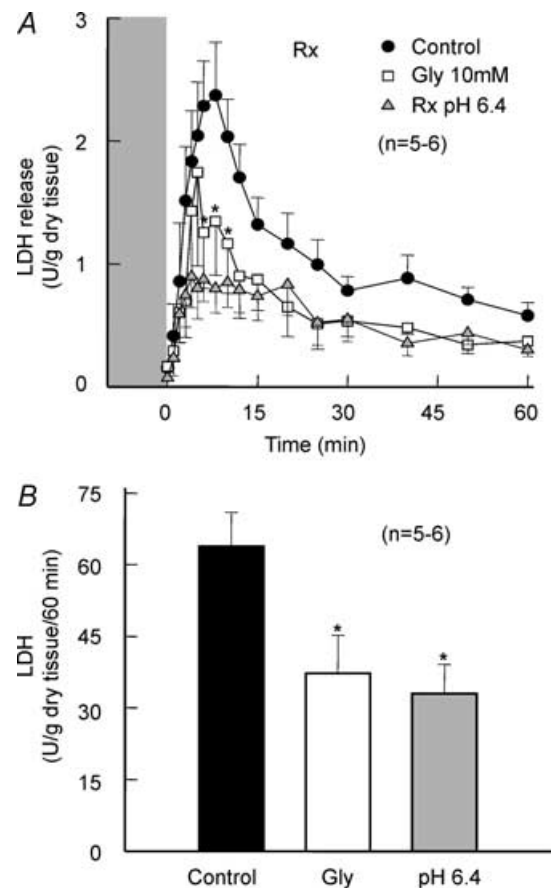
Figure 3. Changes of intracellular pH in cardiomyocytes submitted to 1 h SI–30 min reoxygenation (Rx)

A, intracellular pH immediately before SI (baseline), after 1 h SI and at the end of reoxygenation was not modified by the addition of 3 mM Gly during the reoxygenation period. *B*, original measurements of intracellular H$^+$ concentration (a.u. of BCECF ratio fluorescence) in 2 representative experiments. Addition of Gly did not modify the time course of pH recovery during reoxygenation

Figure 4. Reoxygenation injury in isolated rat hearts

A, reoxygenation-induced LDH release in control and Gly-treated hearts, and in hearts reoxygenated at pH 6.4. *B*, total LDH release during reoxygenation.

or by sequestration of extracellular Ca^{2+} with 2 mM EGTA (Fig. 5*B*). To further assess the potential inhibitory effect of Gly on MPT, normoxic mitochondria were stressed with 200 μM $CaCl_2$, a manoeuvre known to precipitate mitochondrial permeabilization. The minimum concentration of Gly found to be protective against SI–reoxygenation (3 mM) also reduced

mitochondrial swelling induced by high Ca^{2+} in a similar manner as acidic pH (Fig. 5*C* and *D*).

Calcein release in isolated mitochondria

Addition of 200 μM $CaCl_2$ to calcein-loaded mitochondria induced a rapid and abrupt release of the fluorochrom
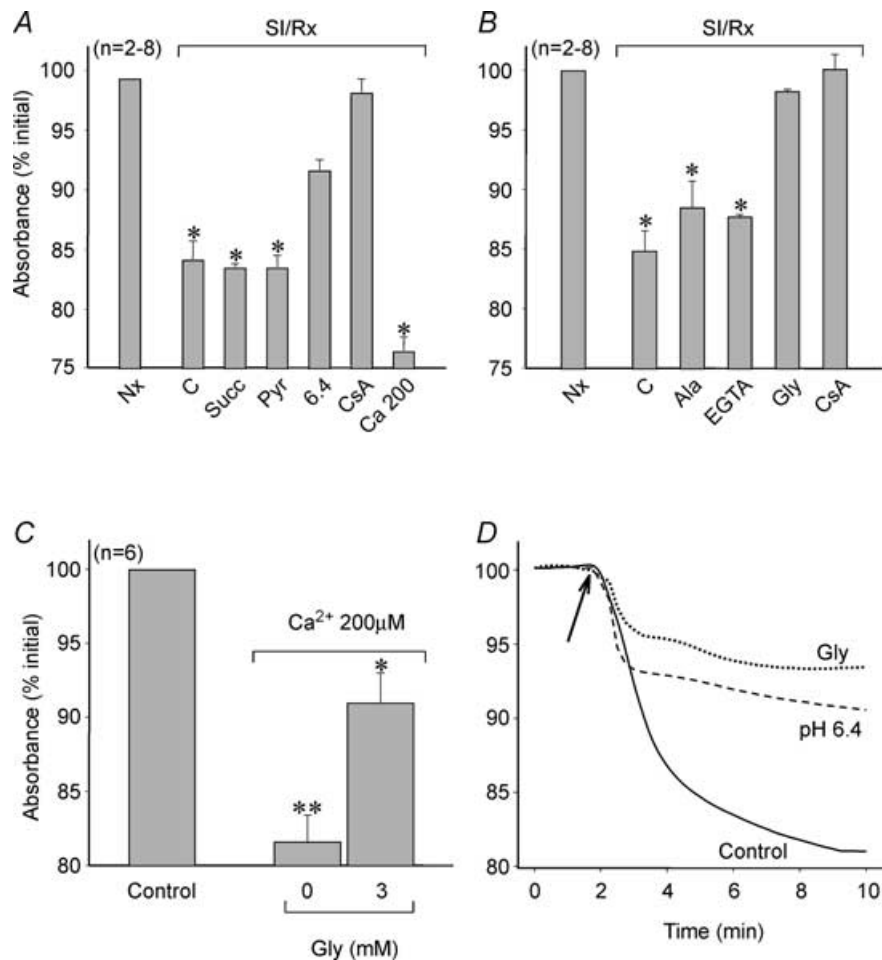

Figure 5. Effect of pH and Gly on mitochondrial swelling
Changes in light absorbance at 520 nm in suspensions of rat heart mitochondria submitted to different experimental protocols. *A*, 30 min of reoxygenation (Rx) after 1 h of SI under the following conditions: at pH 7.2 (C), with 2 different respiratory substrates (5 mM succinate or 5 mM pyruvate), at pH 6.4, with 1 μM CsA or with 200 μM $CaCl_2$. Absorbance is expressed as the percentage value with respect to normoxic mitochondria (Nx). Both acidic reoxygenation (6.4) and CsA prevented the reoxygenation-induced fall in light absorbance, indicative of MPT. *P < 0.05 *versus* Nx. *B*, 30 min of reoxygenation (Rx) after 1 h of SI under the following conditions: at pH 7.2 (C), with 5 mM L-alanine, with 2 mM EGTA, with 3 mM Gly and with 1 μM CsA. Absorbance is expressed as the percentage value with respect to normoxic mitochondria (Nx). Addition of Gly to the reoxygenation buffer was able to completely prevent reoxygenation-induced mitochondrial swelling, as did CsA. *P < 0.05 *versus* Nx. *C*, exposure of normoxic mitochondria to 15 min of 200 μM $CaCl_2$ to promote MPT, with or without 3 mM Gly. Control mitochondria were not submitted to high Ca^{2+} concentration. Addition of 3 mM Gly significantly reduced swelling in Ca^{2+}-stressed mitochondria. *P < 0.05 *versus* Control, **P < 0.05 *versus* 3 mM Gly. *D*, changes in light absorbance throughout time in fully energized mitochondria submitted to 200 μM $CaCl_2$ to promote MPT. The arrows indicate the time at which Ca^{2+} was added. Addition of 3 mM Gly during Ca^{2+} overload was able to prevent mitochondrial swelling more efectively than acid pH.

into the extramitochondrial space (Fig. 6). When Gly was added at the concentrations used to prevent mitochondrial swelling (3 mM or higher), Ca^{2+}-induced calcein release was completely inhibited (Fig. 6). Release of calcein clearly reflected MPT since it could be blocked by CsA (0.1–1 μM).

Intracellular Gly content

NMR spectroscopy revealed that SI–reoxygenation induced a significant reduction of intracellular Gly content. In contrast, a marked increase in intracellular Gly concentration was observed after 30 min reoxygenation when reoxygenation buffer contained 3 mM Gly (Fig. 7).

Discussion

The present study shows that the amino acid Gly has a powerful and previously unrecognized inhibitory effect on MPT, similar to that of low pH, in rat heart mitochondria. We also demonstrate for the first time that addition of 3–10 mM Gly during reoxygenation prevents acute necrotic cell death associated with pH normalization in cultured cardiac myocytes and LDH release in isolated rat hearts. Moreover, exposure to Gly during reoxygenation not only reverses cellular Gly depletion observed in cells submitted to SI–reoxygenation, but markedly raises intracellular Gly content up to the range proven to

inhibit MPT in isolated mitochondria. Altogether, these results indicate that Gly protects cardiomyocytes from reoxygenation-induced cell death by preventing MPT associated with pH normalization.

MPT contributes to cardiomyocyte death induced by normalization of pH during reoxygenation

Disruption of the mitochondrial inner membrane by MPT triggers cell death by different pathways, including the release of cytochrome *c* and other proapoptotic factors that activate caspases and initiate apoptosis (Zamzami & Kroemer, 2001; De Giorgi *et al.* 2002). Less known, but increasingly recognized, is the contribution of MPT to acute necrotic cell death through impaired ATP synthesis and alterations in cation, in particular of Ca^{2+}, homeostasis (Crompton, 1999; Kim *et al.* 2003).

Growing evidence indicates that MPT may contribute to myocardial reperfusion or reoxygenation injury. During prolonged ischaemia, the rapid and profound fall in intracellular pH exerts a powerful inhibitory effect on MPT by antagonizing Ca^{2+} binding to the adenine nucleotide translocase (ANT; Halestrap, 1991). Upon reperfusion, the protective inhibitory effect of low pH is abrogated by the rapid correction of intracellular acidosis, while exacerbated oxidative stress and persistent abnormalities of Ca^{2+} handling may stimulate MPT.
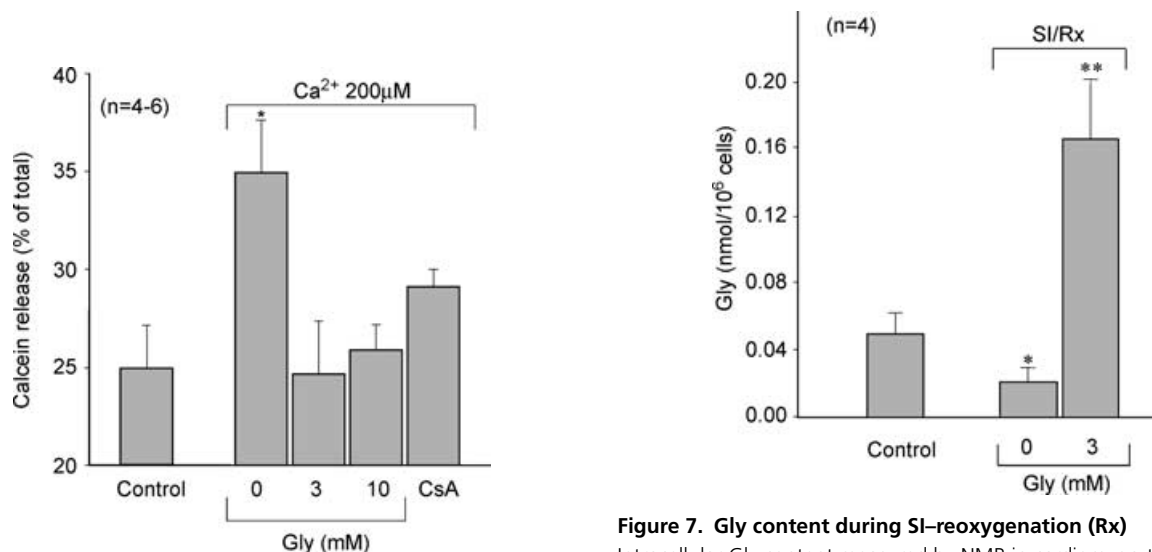

Figure 6. Effect of Gly on mitochondrial calcein release
Calcein release from rat heart mitochondria submitted to 15 min of 200 μM $CaCl_2$ to promote MPT, in the presence of Gly at 2 different concentrations, in its absence, and when MPT was blocked with 0.1 μM CsA. Control group corresponds to mitochondria not submitted to Ca^{2+} overload. Gly 3 mM and 10 mM prevented calcein release, as did CsA. $P < 0.05$ *versus* control.

Figure 7. Gly content during SI–reoxygenation (Rx)
Intracellular Gly content measured by NMR in cardiomyocytes submitted to 1 h SI–30 min reoxygenation in the presence (Gly^+) or absence (Gly^-) of Gly in the reoxygenation buffer. Control group corresponds to cells maintained under Gly-free normoxic conditions. SI–reoxygenation resulted in a reduction of intracellular Gly content while addition of 3 mM Gly to the reoxygenation buffer induced a significant increase in the intracellular Gly content. $P < 0.05$ *versus* normoxic cells, $^{**}P < 0.01$ *versus* normoxic cells.

Experimental evidence of MPT during reperfusion has been provided by fluorescence microscopy of cardio-myocytes re-energized after SI showing CsA-sensitive mitochondrial depolarization (Duchen *et al.* 1993) and calcein loading (Petronilli *et al.* 1999). In reperfused myocardium, MPT has been demonstrated by the loss of mitochondrial NAD^+ (Di Lisa *et al.* 2001) and mitochondrial entrapment of [^{3}H]deoxyglucose 6-phosphate (Javadov *et al.* 2000), with a time course that reflects that of pH normalization.

In the present study, acidic reoxygenation markedly attenuated cell death in cultured HL-1 myocytes and in rat hearts. The protective effect of low pH was independent of the inhibitory effect of acidosis on contractility (since HL-1 cells have only a rudimentary contractile machinery and do not hypercontract upon reoxygenation), was not associated with attenuated cytosolic Na^+ overload, and was not modified by elimination of extra or intracellular Ca^{2+}. The protective effect of acidic pH cannot be thus explained by attenuation of Ca^{2+} overload or hypercontracture, but it rather appears to be related to its inhibitory effect on MPT, as observed in isolated mitochondria.
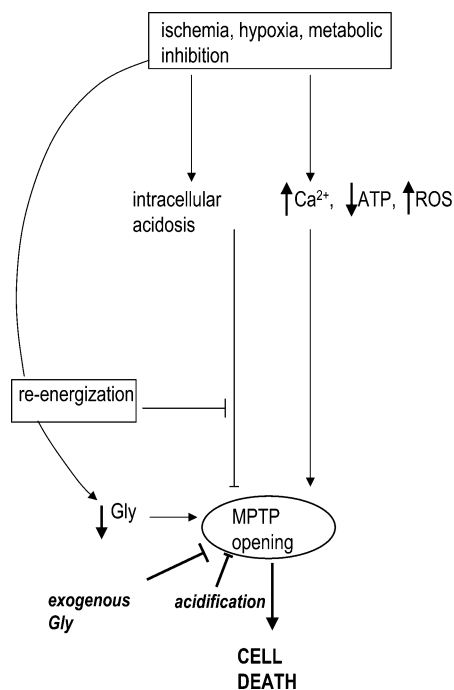

Figure 8. Proposed role of Gly during reperfusion injury
Ischaemia and ischaemia-related conditions are associated with cytosolic derangements that favour MPT, but also with acidosis that prevents it. Reoxygenation rapidly corrects intracellular acidosis, thus allowing MPT, which is facilitated by depletion of intracellular Gly (probably through volume regulatory decrease). Treatment with Gly at the time of reoxygenation inhibits MPT despite correction of acidosis, and prevents cell death.

Gly prevents reoxygenation injury by interfering with MPT

The direct protective effect of Gly against necrotic and apoptotic cell death induced by different insults, including ischaemia–reperfusion, and a variety of other pathological conditions – i.e. endotoxaemic shock, alcoholic hepatitis, hepatic fibrosis, arthritis, tumour and drug toxicity – has been characterized in detail (Zhong *et al.* 1996; Nishimura *et al.* 1998; Nishimura & Lemasters, 2001; Dong *et al.* 2001; Zhang *et al.* 2003). Gly delays sarcolemmal rupture induced by hypoxia, which was initially explained by attenuated Na^+ accumulation obscurely related to stimulation of the Gly-gated Cl^- channels, originally described in the postsynaptic membranes of the spinal cord (Carini *et al.* 2000). Other authors suggested that the protective effect of Gly was independent of receptor stimulation but rather related to the closure of non-specific leaks in plasma membranes induced by hypoxia (Dong *et al.* 1998; Frank *et al.* 2000; Nishimura & Lemasters, 2001). Confocal microscopy analysis of membrane permeabilization to fluorescent molecules of different sizes demonstrated that Gly delays membrane permeabilization during hypoxia, which could reflect the opening of large anionic channels ('death pores') eventually leading to massive cell swelling and sarcolemmal rupture (Nishimura & Lemasters, 2001). Previous studies were coincident in interpreting that Gly prevented plasma membrane failure, the final event in the pathway to necrotic cell death (Frank *et al.* 2000).

In the present study Gly completely prevented cell death associated with normalization of pH during reoxygenation in cardiomyocyte cultures, and mimicked the protective effect of acidic reoxygenation on LDH release in isolated rat hearts. Since acute cell death observed during reoxygenation in our model was ascribable to MPT, we hypothesized that Gly could act at the mitochondrial level, interfering with MPT. The experiments in isolated mitochondria demonstrated that Gly indeed prevents CsA-sensitive mitochondrial swelling and calcein release induced by re-energization after a transient SI or by exposure to high Ca^{2+}. The protective effect of Gly is not related to its utilization as a respiratory substrate or as a source of NAD(P)H, since it was not mimicked by other substrates (succinate or pyruvate) and was present in fully energized mitochondria submitted to Ca^{2+} overload.

The inhibitory effect of Gly on MPT was equivalent to that of low pH (6.4) and was observed at high concentrations (3 mM). Intracellular Gly concentration, measured by NMR spectroscopy, was reduced in HL-1 cells exposed to SI–reoxygenation, as compared to normoxic

cells. Exposure to 3 mM Gly during reoxygenation raised intracellular Gly concentration up to the range proven to inhibit MPT. Measured intracellular content corresponds to an estimated Gly concentration of approximately 7 mM, compared to approximately 1 mM in normoxic cells and 0.6 mM in cells reoxygenated without Gly.

Hypothetical role of Gly depletion in reoxygenation

The mechanism of Gly depletion in cardiomyocytes submitted to SI–reoxygenation was not investigated in the present study. However, amino acid and Cl^- release play an important role in regulating volume decrease in swollen cells (Rasmusson *et al.* 1993; Song *et al.* 1998). It should be thus expected that the rapid reduction in cell volume occurring during reoxygenation and normalization of intracellular pH results in Gly efflux. Although the contribution of Gly efflux to volume decrease has not been established, its abolition by exposure to increased Gly concentration in the extracellular medium should reduce it and hamper cell volume correction. In this study, addition of Gly to the reoxygenation buffer attenuated volume decrease, and this effect was closely correlated with the magnitude of the protection afforded against cell death.

A hypothesis that integrates the results obtained in the present study is outlined in Fig. 8. Rapid pH normalization during reoxygenation favours MPT and cell death. Intracellular Gly acts as an inhibitor of MPT. However, volume regulatory decrease occurring upon normalization of extracellular pH involves cellular Gly loss, which makes the cell more vulnerable to MPT, an effect that can be reversed by increasing extracellular Gly concentration.

The contribution of Gly to cardiomyocyte survival after an ischaemic insult represents a promising therapeutic approach to preventing cell death in reperfused myocardium. Studies in volunteers have shown that plasmatic Gly concentrations higher than those found to be protective in this study can be rapidly achieved by intravenous infusion, and are well tolerated (Nilsson *et al.* 1996).

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