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Cardiovascular Research 60 (2003) 617–625
Cardiovascular Research
www.elsevier.com/locate/cardiores

# Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia–reperfusion injury

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Received 6 January 2003; accepted 10 September 2003

**Time for primary review 24 days**

**Abstract**

**Objective:** The opening of the mitochondrial permeability transition pore (mPTP) in the first few minutes of post-ischaemic reperfusion is a critical determinant of reperfusion-induced cell death. We hypothesised that the novel immunosuppressant, sanglifehrin-A (SFA), given at the time of reperfusion, protects the myocardium from ischaemia–reperfusion injury, by suppressing mPTP opening. **Methods:** Isolated perfused rat hearts were subjected to 35 min ischaemia/120 min reperfusion, and were treated with (1) SFA (1.0 μM) or (2) DMSO vehicle for the first 15 min of reperfusion or (3) SFA (1.0 μM) after the first 15 min of reperfusion. We examined the effect of SFA on mPTP opening directly, using a myocyte model of oxidative stress. Laser illumination of adult rat myocytes loaded with the fluorophore, TMRM, generates oxidative stress, which induces mPTP opening (represented by mitochondrial membrane depolarisation) followed by rigour contracture. **Results:** In the isolated perfused heart model, SFA, given *during* the first 15 min of post-ischaemic reperfusion, reduced the infarct-risk volume ratio from 43.9 ± 2.5% in the control group to 23.8 ± 4.2% with SFA ($p = 0.001$). However, when SFA was given *after* the first 15 min of reperfusion, there was no change in infarct size (43.8 ± 5.7% with SFA vs. 43.9 ± 2.5% in control; $p$ = NS), suggesting that SFA has to be present during the first 15 min of reperfusion to induce protection. In the isolated adult myocyte model, SFA was shown to inhibit mPTP opening in the setting of oxidative stress, represented by an increase in the ROS threshold required to induce: mitochondrial membrane depolarisation (from 269 ± 21 to 777 ± 100 s; $p < 0.001$) and rigour contracture (from 613 ± 14 to 1329 ± 129 s; $p < 0.001$). **Conclusions:** Inhibiting mPTP opening during the first few minutes of reperfusion, using sanglifehrin-A, limits infarct size and protects myocytes from oxidative stress.
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*Keywords:* Infarction; Ischaemia; Membrane permeability; Membrane potential; Reperfusion

## 1. Introduction

Reperfusion is a pre-requisite for salvaging viable myocardium from an episode of prolonged myocardial ischaemia. Paradoxically, the act of reperfusion is not without risk, as it can in itself, induce further myocyte death—a phenomenon termed lethal reperfusion-induced injury [1]. The concept of reperfusion-induced injury as an independent determinant of cell death is unresolved, with some studies suggesting that reperfusion only exacerbates the cellular injury sustained during the ischaemic period [2,3]. Other studies, on the other hand, indicate that the oxidative stress and abrupt metabolic changes that accompany reperfusion can themselves initiate cellular injury in the absence of ischaemia [4,5]. The most convincing evidence for the existence of reperfusion-induced injury as a distinct entity is provided by the observation that myocyte survival can be influenced by interventions applied solely at the time of post-ischaemic reperfusion, implying that crucial events which occur at time of reperfusion can be modified.

In this regard, a critical determinant of cell death in the setting of ischaemia–reperfusion injury is the opening of the mitochondrial permeability transition pore (mPTP) [6]. By opening in the first few minutes of reperfusion, the mPTP mediates reperfusion-induced myocyte death [7,8]. The

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doi:10.1016/j.cardiores.2003.09.025

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mPTP is a large conductance pore of the inner mitochondrial membrane, which on opening renders the otherwise impermeable inner membrane, freely permeable to solutes up to 1500 Da in size [6,9]. This results in swelling of the mitochondrial matrix and rupture of the outer mitochondrial membrane, leading to the translocation of cytochrome *C* and other pro-apoptotic factors into the cytosol, which initiate the apoptotic death pathway [10]. Furthermore, the opening of the mPTP uncouples mitochondrial oxidative phosphorylation, resulting in the collapse of the mitochondrial membrane potential, which leads to cellular death by necrosis if the mPTP opening is prolonged and ATP levels are depleted [6].

In the setting of ischaemia–reperfusion injury, the mPTP is believed to open in the first few minutes of reperfusion, when conditions that increase its opening probability prevail; these include a high mitochondrial calcium and inorganic phosphate load, ATP depletion, oxidant stress and a corrected matrix pH [7,8,11]. We and others have shown that inhibiting its opening at the time of reperfusion using the immunosuppressant cyclosporin-A (CsA) protects the myocardium from ischaemia–reperfusion-induced injury [12,13]. Although CsA is a potent inhibitor of mPTP opening, it also has other actions [14], which include inhibiting the phosphatase, calcineurin [15].

Recently, the novel immunosuppressant, sanglifehrin-A (SFA), has been shown to also act as a potent inhibitor of mPTP opening [16]. This drug has been demonstrated to be a more potent inhibitor of mPTP opening than CsA and, in addition, SFA does not inhibit calcineurin [16,17]. When administered prior to the lethal ischaemic period in the isolated perfused heart, SFA was shown to improve recovery of LV function and reduce LDH release [16]. However, its action at the crucial time of reperfusion alone, when the mPTP is believed to open, has never been studied.

As such, the aim of this study was to investigate the effect of SFA at reperfusion, in the isolated perfused rat heart using infarct size as the end-point of injury, to ascertain its ability to protect the myocardium against the consequences of lethal reperfusion-induced injury. Furthermore, in order to demonstrate the mechanism by which SFA may induce protection, we used a myocyte model for detecting mPTP opening in isolated adult rat ventricular myocytes subjected to oxidative stress.

## 2. Methods

### 2.1. Animals

Male Sprague–Dawley rats ($n = 23$, $300 \pm 50$ g body weight) were used. All animals were obtained from Charles River UK (Margate, UK) and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationery Office, London, UK). The investigation conforms with the *Guide for the Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.2. Isolated heart perfusion

Rats were anaesthetised with sodium pentobarbital (55 mg/kg intraperitoneally) and given heparin sodium (300 IU). Hearts were rapidly excised and placed in ice-cold buffer and mounted on a constant pressure (100 mm Hg) Langendorff-perfusion apparatus. They were perfused retrogradely with modified Krebs–Henseleit bicarbonate buffer containing (in mM): NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 1.7 and glucose 12.0. All solutions were gassed with 95% $O_2$/5% $CO_2$ with pH maintained between 7.35 and 7.45 at 37 °C. Temperature was continuously monitored by a thermo-probe inserted into the pulmonary artery and maintained between 36.5 and 37.5 °C. A latex, fluid-filled, isovolumic balloon was introduced into the left ventricle through the left atrial appendage and inflated to give a pre-load of 8–10 mm Hg. Left ventricular developed pressure, heart rate and coronary flow were measured at regular intervals. A surgical needle was passed under the left main coronary artery, and the ends of the suture were passed through a pipette tip to form a snare. Tightening the snare induced regional ischaemia and releasing the ends of the suture initiated reperfusion [13].

### 2.3. Treatment protocols for infarct study

The experimental protocols for the infarct studies are presented in Fig. 1. All hearts received 35 min regional ischaemia and 120 min reperfusion. SFA (Novartis Pharma, Basel) was dissolved in dimethyl sulphoxide (DMSO) and added to the Krebs–Henseleit buffer such that the final DMSO concentration was less than 0.01%. The hearts were randomly assigned to one of the following treatment groups: (1) control hearts were perfused with 0.01% DMSO ($n = 6$) for the first 15 min of reperfusion; (2) hearts ($n = 6$) were perfused with SFA (1.0 μM) for the first 15 min of reperfusion. This concentration of SFA has been shown to give the most potent inhibition of mPTP opening [3,16]. Hearts ($n = 5$) were perfused with SFA (1.0 μM) after the first 15 min of reperfusion. This group was included to determine whether SFA would have any effect if given after the crucial first 15 min of reperfusion, the time period during which the mPTP has been demonstrated to open [7,8].

### 2.4. Infarct size measurement

At the end of the 120 min reperfusion period, the snare was pulled tight and the heart was slowly perfused with

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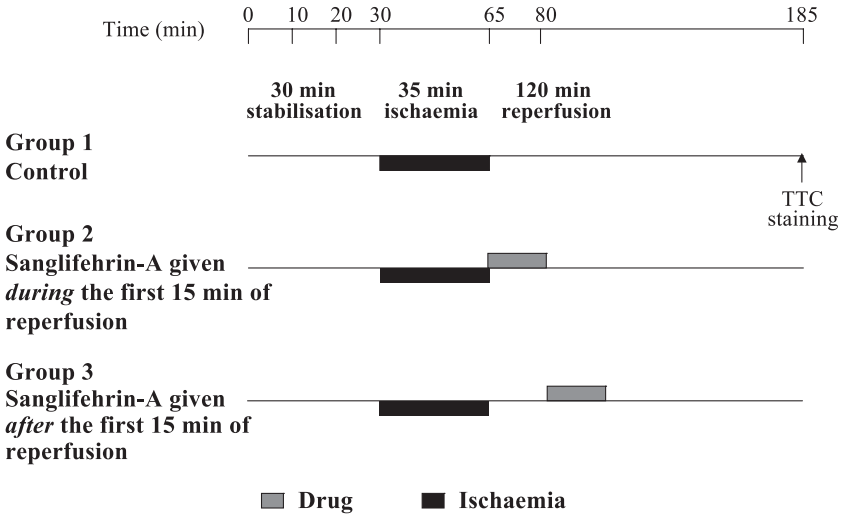


Fig. 1. Treatment protocols for the infarct studies. TTC—triphenyltetrazolium chloride.

saline solution containing 0.25% Evans blue dye, to delineate the non-ischaemic zone of the myocardium as a dark blue area. After 1–4 h at −20 °C, the hearts were sliced into 2-mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37 °C for 10–15 min. The tissue slices were then fixed in 10% formalin. In the risk zone, the viable tissue was stained red and the infarcted tissue appeared pale. The slices were drawn onto acetate sheets and with the use of a computerised planimetry package (Summa Sketch III, Summagraphics, Seymour, CT, USA); the percentage of infarcted tissue within the volume of myocardium at risk was calculated [13].

### 2.5. Preparation of adult rat myocytes

Adult rat myocytes were isolated by collagenase perfusion using a previously described method with modifications [18]. Briefly, following anaesthesia with sodium pentobarbital (55 mg/kg intraperitoneally) and the administration of heparin sodium (300 IU), hearts were rapidly excised and placed in ice-cold buffer, and mounted on a non-recirculating perfusion apparatus. All solutions used were based on a modified calcium-free Krebs–Ringers–Hepes (KRH) buffer (in mM) NaCl 116.0, KCl 5.4, $MgSO_4$ 0.4, HEPES 20.0, $Na_2HPO_4$ 0.9, glucose 5.6 (pH 7.4). The perfusate was bubbled with 100% $O_2$ and maintained at 37 °C. Hearts were first perfused at 14 ml/min with KRH buffer containing 1 mg/ml BSA and 3.3 μM EGTA. After 5 min, the hearts were switched to KRH buffer containing 0.75 mg/ml collagenase (Worthington type II) and 25 μM calcium for 10–15 min. They were finally perfused with KRH buffer containing 50 μM calcium for 5 min. Following perfusion, the hearts were removed from the perfusion apparatus and the atria trimmed away. The ventricles were minced and underwent several more digestions with collagenase. The cells were then filtered through a nylon mesh and washed with restoration buffer: KRH buffer + 10 mg/ml BSA, 0.5 mM Na pyruvate, 5.0 mM taurine, 2.0 mM carnitine, 1.0 mM creatine and 75 μM calcium. Next, the calcium concentration was gradually increased to 1.25 mM. After isolation, the cells were seeded onto sterilised laminin-coated 25 mm diameter round cover-slips and incubated overnight at 37 °C in an atmosphere of 95% air/5% $CO_2$ in M-199 medium (M7653, Sigma) containing 10% foetal calf serum and 1% penicillin–streptomycin (Sigma). The next day, the cells were washed and kept in restoration buffer for the duration of the experiments.

### 2.6. Model for detecting mPTP opening in the intact cell

In order to open the mPTP routinely and predictably, we have used controlled intra-mitochondrial oxidative stress and have used mitochondrial membrane depolarisation and the rigor contracture that follows the resultant ATP depletion as endpoints. The fluorescent dye TMRM was used: (a) to report mitochondrial membrane potential, with global mitochondrial depolarisation signalling mPTP opening, and (b) to act as a photosensitising agent to generate the oxidative stress on illumination required to induce mPTP opening followed by rigour contracture [19–24]. Laser-induced oxidative stress was continued until mPTP opening had been provoked, and then continued until rigour contracture took place. This model represents a widely reported and reliable way to reproducibly induce the loss of mitochondrial potential, which has been unequivocally identified as a reflection of mPTP opening [19–24]. TMRM, a lipophilic cation, accumulates selectively into mitochondria according to the mitochondrial membrane potential. On laser-induced photo-sensitisation, TMRM generates reactive oxygen species (ROS) from within the mitochondria, which provoke mPTP opening [19–22,24]. As the oxidative stress gener-

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ated at reperfusion also involves excess production of radical species from mitochondria, this should represent a useful model for reoxygenation-induced cell injury. Cells were incubated with TMRM (3 μM) for 15 min at 37 °C followed by washing. Opening of the mPTP results in mitochondrial membrane depolarisation, which is detected in this model as an increase in TMRM fluorescence intensity. The relatively high concentration of TMRM in the mitochondria causes auto-quenching of fluorescence, so that the fluorescence signal becomes a non-linear function of dye concentration; therefore, mitochondrial depolarisation results in loss of dye into the cytosol where the signal increases [25].

Cells were assigned to: (1) control-incubation with 0.01% DMSO vehicle control for 20 min ($n = 12$); (2) incubation with sanglifehrin-A (1.0 μM, $n = 12$) for 20 min. We measured the time taken to the beginning of global mitochondrial depolarisation (represented by an increase in cytosolic TMRM fluorescence and indicates mPTP opening) and the time taken to the beginning of irreversible cell shortening (rigor contracture, signalling ATP depletion).

### 2.7. Confocal imaging and analysis

The cover-slip with adherent myocytes was placed in a chamber and mounted on the stage of a Zeiss 510 CLSM confocal microscope equipped with ×40 oil immersion, quartz objective lens (NA 1.3). The cells were illuminated using the 543-nm emission line of a HeNe laser. For all photo-sensitisation experiments, all conditions of the confocal imaging system (laser power, confocal pinhole—set to give an optical slice of 1 μm—pixel dwell time and detector sensitivity) were identical to ensure comparability between experiments. The fluorescence of TMRM was collected using a 585-nm long pass filter. Images were analysed using the Zeiss software (LSM 2.8).

### 2.8. Statistical analysis

All values are expressed as mean ± S.E.M. Infarct size and times taken to induce global mitochondrial depolarisation and irreversible cell shortening were analysed by one-way ANOVA and Fisher's protected least significant difference test for multiple comparisons. Rate pressure product and coronary flow were analysed by a two-way ANOVA for repeated measures. Differences were considered significant when $p < 0.05$.

## 3. Results

### 3.1. Isolated perfused rat heart model

Baseline data relating to cardiac function (assessed by the rate-pressure-product, RPP) and coronary flow before the lethal ischaemic period, were similar in the experimental groups (see Table 1). During regional ischaemia, coronary flow and RPP decreased to a similar extent in both groups. An increase in cardiac function and coronary flow upon reperfusion was indicative of successful re-flow. Perfusion with SFA for the first 15 min of reperfusion had no immediate effect on cardiac function and coronary flow compared to control. However, at the end of the reperfusion period, hearts treated with SFA for the first 15 min of reperfusion had significantly better cardiac function (RPP of 17.5 ± 2.4 mm Hg/min with SFA vs. 8.3 ± 2.3 mm Hg/min in control; $p < 0.005$) and coronary flow (9.2 ± 0.9 ml/

Table 1
Haemodynamic variables throughout the ischaemia–reperfusion protocol

| Groups | 15-min stabilisation | 30-min ischaemia | 15-min reperfusion[a] | 120-min reperfusion | Risk volume ($cm^3$) | Infarct volume ($cm^3$) | Infarct-risk (%) |
|---|---|---|---|---|---|---|---|
| Control | | | | | 0.411 ± 0.017 | 0.184 ± 0.011 | 43.9 ± 2.5 |
| Heart rate (bpm) | 308 ± 15 | 246 ± 22 | 278 ± 20 | 228 ± 36 | | | |
| LVDP (mm Hg) | 91.0 ± 14.9 | 54.3 ± 7.8 | 75.2 ± 8.2 | 36.4 ± 8.5 | | | |
| RPP × $10^3$ (mm Hg/min) | 28.0 ± 5.8 | 12.8 ± 3.1 | 20.9 ± 3.3 | 8.3 ± 2.3 | | | |
| Coronary flow (ml/min) | 17.3 ± 0.5 | 7.2 ± 0.3 | 10.0 ± 1.5 | 5.3 ± 0.3 | | | |
| Sanglifehrin-A | for the first 15 min of reperfusion | | | | 0.485 ± 0.046 | 0.115 ± 0.021* | 23.8 ± 4.2* |
| Heart rate (bpm) | 330 ± 12 | 258 ± 11 | 254 ± 12 | 276 ± 4 | | | |
| LVDP (mm Hg) | 88.6 ± 10.9 | 59.2 ± 7.6 | 70.2 ± 11 | 63.6 ± 9.1** | | | |
| RPP × $10^3$ (mm Hg/min) | 28.9 ± 2.9 | 15.5 ± 2.5 | 18.2 ± 3.5 | 17.5 ± 2.4** | | | |
| Coronary flow (ml/min) | 17.2 ± 1.8 | 7.4 ± 0.7 | 10.6 ± 1.3 | 9.2 ± 0.9* | | | |
| Sanglifehrin-A | after the first 15 min of reperfusion | | | | 0.505 ± 0.033 | 0.221 ± 0.029 | 43.8 ± 5.7 |
| Heart rate (bpm) | 278 ± 16 | 258 ± 19 | 234 ± 13 | 228 ± 2 | | | |
| LVDP (mm Hg) | 94.0 ± 7.4 | 55.8 ± 4.5 | 86.0 ± 8.8 | 47.5 ± 5.5 | | | |
| RPP × $10^3$ (mm Hg/min) | 25.4 ± 3.0 | 12.1 ± 1.8 | 17.2 ± 1.6 | 11.5 ± 0.6 | | | |
| Coronary flow (ml/min) | 16.2 ± 1.6 | 7.0 ± 1.0 | 14.4 ± 0.9 | 6.6 ± 0.8 | | | |

Values are mean ± S.E.M. RPP, rate-pressure product; LVDP, left ventricular developed pressure.
[a] Immediately following 15 min administration of sanglifehrin-A.
* $p < 0.005$, compared with control.
** $p < 0.05$, compared with control.

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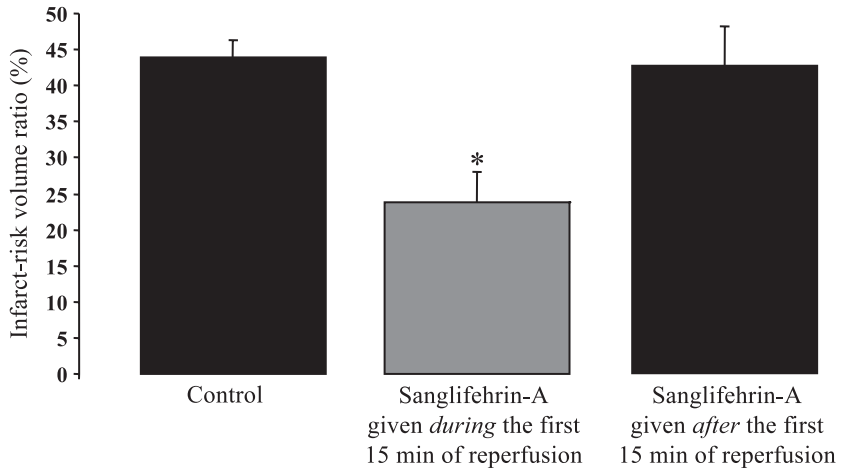


Fig. 2. Sanglifehrin-A given *during* the first 15 min of reperfusion reduces the infarct-risk volume ratio, but not when given *after* the first 15 min of reperfusion (values are mean ± S.E.M.; $n = 5–6$ per group; $*p = 0.001$).

min with SFA vs. 5.3 ± 0.3 ml/min in control; $p < 0.05$; Table 1). In hearts treated with SFA for the first 15 min of reperfusion, the % recovery of baseline cardiac function after 120 min of reperfusion was 60.6% with SFA vs. 29.6% in control hearts ($p < 0.005$). However, in hearts perfused with SFA given after the first 15 min of reperfusion, compared to control, there was no difference in either cardiac function (RPP of 11.5 ± 0.6 mm Hg/min with SFA given after the first 15 min of reperfusion vs. 8.3 ± 2.3 mm Hg/min in control; $p$ = NS) or coronary flow (6.6 ± 0.8 ml/min with SFA given after the first 15 min of reperfusion vs. 5.3 ± 0.3 ml/min in control; $p$ = NS; Table 1) at the end of the 120 min of reperfusion.

The risk zone volumes were similar in the experimental groups (0.411 ± 0.017 $cm^3$ in control vs. 0.485 ± 0.046 $cm^3$ with SFA given for the first 15 min of reperfusion vs. 0.505 ± 0.046 $cm^3$ with SFA given after the first 15 min of reperfusion; $p$ = NS). Infarct size was represented as the

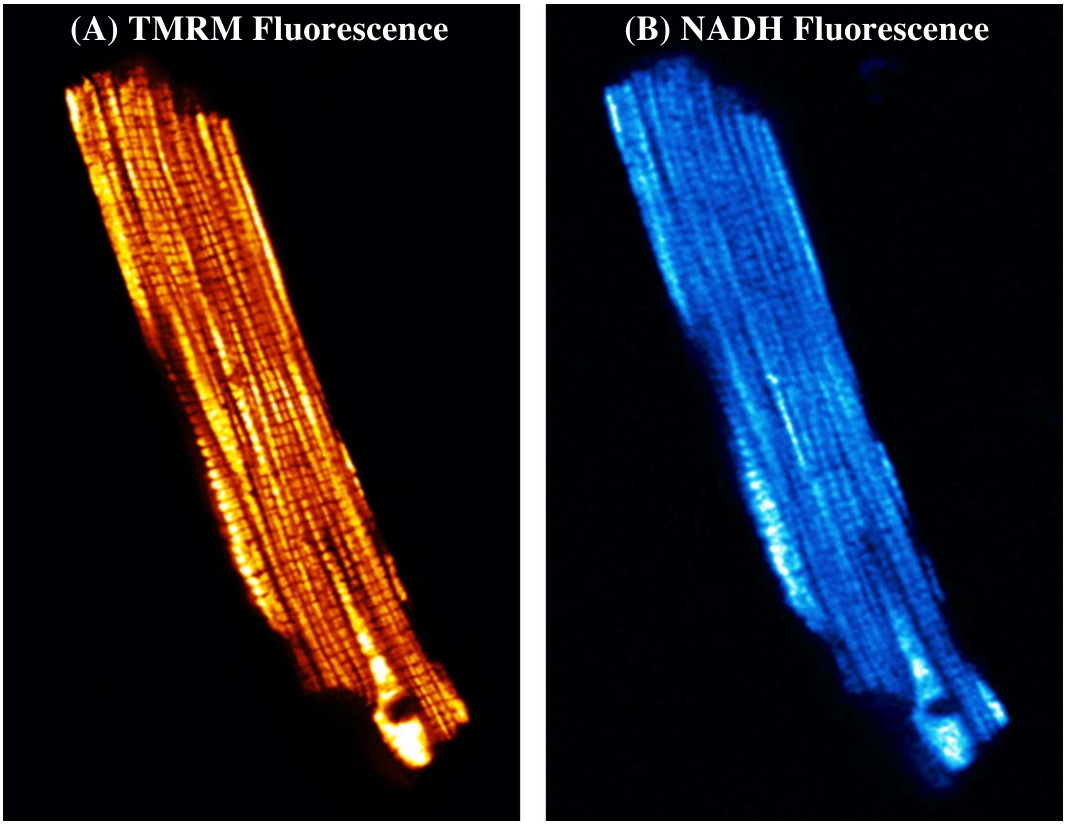


Fig. 3. Confocal image of representative myocyte showing co-localisation of (A) TMRM fluorescence and (B) NADH fluorescence within the mitochondria.

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percentage of tetrazolium-negative tissue in the ischaemic risk zone. The presence of SFA for the first 15 min of reperfusion significantly reduced infarct size compared with control hearts (23.8 ± 4.2% with sanglifehrin-A vs. 43.9 ± 2.5% in control; $p < 0.01$; Fig. 2). However, when SFA was omitted for the first 15 min of reperfusion and only given after 15 min of reperfusion had elapsed, there was no influence on infarct size (43.8 ± 5.7% with SFA given after the first 15 min of reperfusion vs. 43.9 ± 2.5% in control; $p$ = NS; Fig. 2), which suggests that SFA needs to be present during the first 15 min of reperfusion to exert its cardioprotective effect.

### 3.2. Model for detecting mPTP opening in intact cells

Confocal imaging of TMRM-loaded adult ventricular myocytes revealed bands of intracellular fluorescence throughout the cell. These bands of fluorescence correspond to mitochondria, and their identity is confirmed by their co-localisation with mitochondrial NADH autofluorescence (Fig. 3).

Fig. 4i–v shows a representative TMRM-loaded myocyte, subjected to oxidative stress, and demonstrates the sequential changes in mitochondrial membrane potential. With initial oxidative stress, one observes, depolarisation of individual mitochondria (which appear as areas devoid of TMRM fluorescence, see arrow in Fig. 4ii). Of interest, with continued oxidative stress, global mitochondrial membrane depolarisation, which reflects mPTP opening, begins as a wave of increased cytosolic TMRM fluorescence at one end of the cell (see arrow in Fig. 4iii) and progresses across the full length of the cell. The cause of this wave of global mitochondrial depolarisation is not known but this phenomenon may reflect a self-propagating wave of mPTP opening in mitochondria, with calcium released from one mitochondria on mPTP opening, initiating mPTP opening in the adjacent mitochondria and so on, [26,27] leading to global mitochondrial depolarisation of the whole cell (Fig. 4iv). As oxidative stress continues, rigour contracture occurs, signifying ATP depletion (Fig. 4v). Therefore, using this model of oxidative stress, we measured the time taken to induce the initiation of global mitochondrial membrane depolarisation (which represents mPTP opening) and the time taken to induce the initiation of rigor contracture (representing ATP depletion).

The time required to induce mPTP opening (indicated by the beginning of global mitochondrial membrane depolarisation) in control cells was 269.1 ± 21.7 s and the time taken to induce the beginning of rigour contracture was 613.5 ± 14.2 s (Fig. 5). The presence of SFA significantly prolonged the time taken to induce both mPTP opening and rigour contracture to 777.4 ± 100.7 and 1329.0 ± 129.0 s

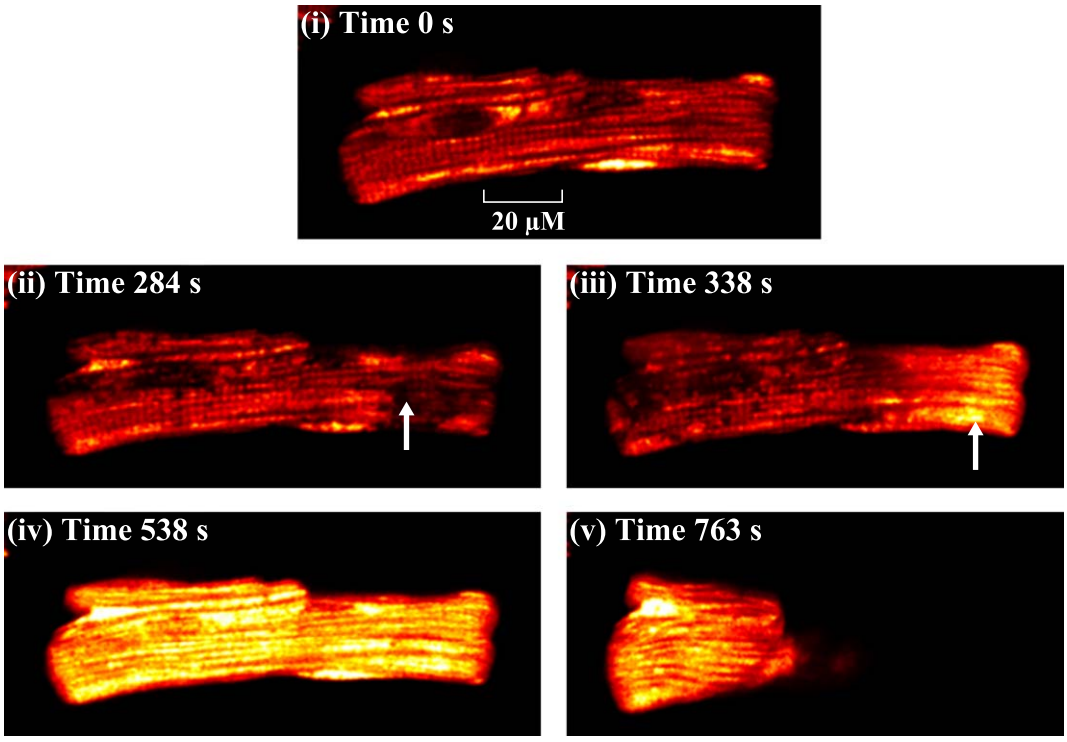


Fig. 4. Confocal image of representative adult ventricular myocyte loaded with TMRM showing the sequential changes on exposure to oxidative stress: (i) the cell prior to oxidative stress showing TMRM localised to the mitochondria as bands of increased red-orange fluorescence; (ii) with continued oxidative stress areas devoid of fluorescence appear (see arrow), which precede (iii) a wave of global mitochondrial membrane depolarisation (appearing as increased cytosolic fluorescence, signalling mPTP opening) that begins at one end of the cell and progresses across the full length of the cell, until the whole cell is globally depolarised; (iv) and finally rigour contracture ensues following ATP depletion (v).

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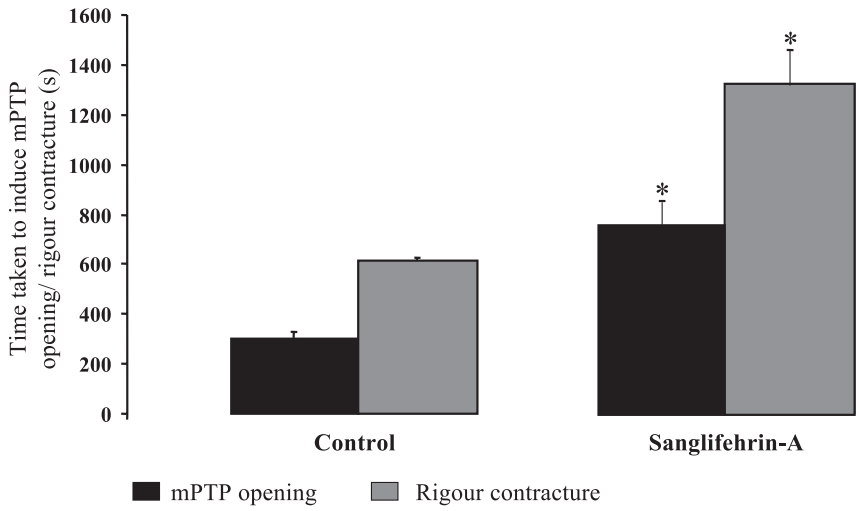


Fig. 5. Sanglifehrin-A prolongs the time taken to induce mPTP opening and the time taken to induce rigour contracture in TMRM-loaded myocytes subjected to oxidative stress (values are mean ± S.E.M.; $n = 12$ per group; $*p < 0.001$).

($p < 0.001$), respectively (Fig. 5). Sanglifehrin-A was therefore shown to inhibit mPTP opening (as shown by a delay in the time taken to induce global mitochondrial membrane depolarisation) and protect against rigour contracture in the setting of oxidative stress.

## 4. Discussion

In the isolated perfused heart model, we show for the first time that inhibiting mPTP opening in the first few minutes of reperfusion, following a lethal ischaemic period, using sanglifehrin-A, protects the myocardium, as shown by a reduction in infarct size and improved recovery of baseline cardiac function. Furthermore, in the isolated adult rat myocyte subjected to oxidative stress, we confirm that sanglifehrin-A protects myocytes by inhibiting mPTP opening.

Importantly, in the isolated perfused heart model, we demonstrated protection when SFA was administered during the first 15 min of reperfusion only, to cover the crucial time period when the mPTP would be expected to open. Furthermore, we demonstrated that targeting the first 15 min of reperfusion is crucial to its protective effect, since administering SFA after the first 15 min of reperfusion induced no cardio-protection. This last finding suggests that SFA has to be present during the first 15 min of reperfusion to exert it protective effect, which corresponds to the time-period during which mPTP opening has been demonstrated to occur [7,8]. Although SFA has been investigated in the setting of ischaemia–reperfusion injury, the drug was administered prior to index global ischaemia and for a further 10 min of reperfusion [16]. Furthermore, in that study, the end-points of post-ischaemic recovery of LV function and LDH release were used. As such, it is difficult to interpret whether this agent was only having its effect during the reperfusion phase, given that the drug was administered prior to the lethal ischaemic episode.

The current proposed model of the mPTP is believed to comprise a physical interaction between three core components: the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane, the adenine nucleotide translocase (ANT) of the inner mitochondrial membrane and cyclophilin D in the mitochondrial matrix [6]. Through its *cis–trans* isomerase action, it is believed that cyclophilin D induces a conformational change in the ANT from its normal translocating configuration to a pore-forming configuration [28]. The mechanism by which SFA inhibits mPTP opening has been shown to differ from that of CsA. The latter appears to inhibit binding of mitochondrial cyclophilin D to the ANT [29], whereas SFA inhibits the peptidyl-prolyl *cis–trans* isomerase activity of cyclophilin D, thereby preventing the conformational change of the ANT to the pore-forming configuration required to manifest as mPTP opening [16]. Furthermore, in contrast to CsA, the drug SFA is a more specific inhibitor of mPTP opening as it does not inhibit calcineurin, which is important as calcineurin inhibition can in itself result in cardio-protection [16,17].

The contribution of the mPTP to ischaemia–reperfusion injury was first proposed in 1987, when it was found that factors, which increase the opening probability of the mPTP, prevailed in the setting of ischaemia–reperfusion injury [30]. These factors included, a rise in tissue $Ca^{2+}$ and iP levels, and depletion of ATP levels during the ischaemic period, followed by a burst of oxidant stress and further influx of calcium at time of reperfusion [30,31]. In agreement with the crucial role of the mPTP in ischaemia–reperfusion injury, inhibiting its opening, using the drug cyclosporin-A, has been shown to protect against necrosis in myocytes exposed to anoxia-reoxygenation [32,33], and protect the myocardium from ischaemia–reperfusion injury in the isolated perfused heart [12,13].

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The actual time when mPTP opening occurs in the setting of ischaemia–reperfusion has been characterised, and studies have shown that the mPTP remains closed during the ischaemic period and only opens in the first few minutes of reperfusion [7,8,34]. It has been suggested that the mPTP remains closed during the ischaemic period, because its opening is inhibited by the lactate-induced acidic conditions. It has previously been shown that mPTP opening is inhibited at pH values below 7 [11,35,36]. At reperfusion, there is a further influx of calcium into the mitochondria, a burst of oxidant stress and rapid correction of the acidosis as the lactate is washed away. All these factors contribute to increasing the opening probability of the mPTP in the early minutes of reperfusion following a lethal ischaemic period.

The first few minutes of reperfusion following a lethal ischaemic insult, therefore, represent a 'window of opportunity' for interventions directed to attenuating reperfusion-induced cell death, via inhibition of mPTP opening. This study and others have shown that pharmacologically inhibiting the mPTP opening that normally occurs at reperfusion protects the myocardium from ischaemia–reperfusion injury in the laboratory setting [12,13]. Further research is needed to apply this approach to the clinical settings of reperfusion, such as during thrombolysis post-myocardial infarction, during coronary angioplasty and during coronary artery bypass surgery, since effective inhibition of mPTP opening in the first few minutes of reperfusion could provide further protection against ischaemia–reperfusion injury.

## Acknowledgements

Dr. Derek Hausenloy is supported by a British Heart Foundation Clinical PhD Studentship (FS/02017). We are grateful to Novartis Pharma (Basel) for providing us with sanglifehrin-A.

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