IL-6 induces PI 3-kinase and nitric oxide-dependent protection and preserves mitochondrial function in cardiomyocytes

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Abstract

Objective: Interleukin-6 (IL-6) is a pro-inflammatory cytokine which is a prognostic marker associated with left ventricular contractile dysfunction and heart failure. On the other hand, IL-6 activates signalling pathways which mediate delayed ischemic preconditioning. We have therefore studied the cellular mechanisms of IL-6-induced cardioprotection.

Methods: Inducible nitric oxide synthase (iNOS) expression, cardiomyocyte calcium handling, mitochondrial energetics, and the activation of protective signalling pathways in response to IL-6 were studied in a model of simulated ischemia/reperfusion (sI/R) in isolated neonatal rat ventricular cardiomyocytes.

Results: Reperfusion after sI/R induced a rise in cytosolic [Ca2+]i, a loss of cell morphology and integrity, and a transient increase in mitochondrial potential (Δψm), followed by mitochondrial swelling and collapse of Δψm. Pre-treatment of cardiomyocytes with 10 ng/ml IL-6 for 6 h, 24 h prior to sI/R prevented the secondary rise in cytosolic [Ca2+]i and induced expression of iNOS and NO-dependent protection against sI/R injury. The protection against sI/R was concomitant with a NO-dependent reduction in the amplitude of cytosolic Ca2+ transients. IL-6 induced an increase in inner mitochondrial membrane polarisation and increased mitochondrial Ca2+ loading (rhod-2 fluorescence) at baseline, but prevented the reperfusion-induced changes in mitochondrial function. IL-6 pre-treatment also resulted in activation of the phosphatidylinositol (PI) 3-kinase/Akt pathway, and both iNOS induction and IL-6-dependent protection were blocked by the PI 3-kinase inhibitor wortmannin.

Conclusion: IL-6 induces a PI 3-kinase and NO-dependent protection of cardiomyocytes, which is associated with alterations in mitochondrial Ca2+ handling, inhibition of reperfusion-induced mitochondrial depolarisation, swelling and loss of structural integrity, and suppression of cytosolic Ca2+ transients.

Keywords: Interleukins; Mitochondria; Calcium; Protein kinase; Nitric oxide

1. Introduction

Mitochondrial Ca2+ ([Ca2+]m) overload is considered a critical parameter in the pathogenesis of cell death on reperfusion following ischemia [1]. During ischemia, cytosolic Ca2+ concentration ([Ca2+]c) increases, and studies to date have suggested that the extent of the subsequent rise in [Ca2+]m at the time of reperfusion determines the likelihood of cell death upon reoxygenation [2]. Mecha-
nisms that limit mitochondrial Ca^{2+} accumulation would be expected to confer protection from reperfusion induced cell injury [3]. Following cardiac ischemia, the depression of cardiac contractility, the development of secondary hypertrophy and eventual heart failure are in part due to alterations in cardiomyocyte calcium handling through NO-dependent depression of cardiomyocyte calcium transients [4] and also downregulation of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) [5].

Interleukin-6 (IL-6) is a pleiotropic, pro-inflammatory cytokine, the circulating levels of which are elevated in patients with congestive heart failure [6] and it is also released from the border zone of myocardial infarcts [7]. Under these conditions IL-6 is thought to play a role in the depression of contractility, the development of hypertrophy and heart failure following ischemia. However, IL-6 has been shown to be cardioprotective with recent evidence supporting a role as an obligatory mediator of delayed ischaemic preconditioning [8]. IL-6 is released from cardiomyocytes under hypoxic conditions [9] and in response to activation of the adenosine A1 receptor [10]. Other IL-6 family cytokines such as cardiотrophin-1 (CT-1) and leukemia inhibitory factor (LIF) have been shown to mediate both cardioprotection and hypertrophy, seemingly via distinct signalling pathways [11–14]. The IL-6 family of cytokines signal through multi-subunit receptor complexes that share a common transmembrane glycoprotein signalling subunit, gp130. Loss of gp130 signalling in cardiomyocytes exacerbates the onset of heart failure in response to pressure overload [15] and therefore represents an essential survival pathway. However, the downstream effectors that are activated by IL-6 itself to mediate the protective effects are still not well understood.

Nitric oxide (NO) is a critical mediator of myocardial homeostasis and the responses to a multitude of stressors and agonists and has been shown to mediate both protective and injurious effects in the heart. In keeping with this apparent complexity, the effects of NO seem to depend on the tissue compartment, the concentration and the time course of production (recently reviewed in Ref. [16]). In this study we have examined the cytoprotective properties of IL-6 at the cellular level in a model of simulated ischaemia in an attempt to gain insights into its mechanism of action.

2. Materials and methods

2.1. Materials

Cell culture media were obtained from Life Technologies (UK). Fluorescent indicator dyes were from Molecular Probes (UK) and collagenase from Worthington (USA). Murine recombinant IL-6 and other general laboratory reagents were from Sigma (UK). Wortmannin was from Calbiochem (UK). Antibodies against the phosphorylated Serine 473 of PKB/Akt were from Cell Signalling Technology (Affinity Bioreagents, UK). Antibodies against iNOS were from BD-Transduction Labs (UK).

2.1.1. Methods

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85–23, revised 1996).

2.2. Isolation and culture of neonatal rat ventricular cardiomyocytes

Primary rat ventricular cardiomyocytes were prepared from 1–2 day old Sprague Dawley rats, essentially as described in Ref. [17]. After 24 h, cells were cultured in maintenance medium (4:1 DMEM: M199, supplemented with 1% FCS) and experiments performed after 2–3 days in culture.

2.3. Dye loading

Cardiomyocytes were loaded with fluorescent dyes in control buffer (156 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM KH2PO4, 2 mM CaCl2, 10 mM HEPES, 10 mM d-glucose, pH7.35), unless otherwise indicated, as follows: Fura-2 (AM ester): 5 μM with 0.005% pluronic for 30 min at room temperature. TetramethylRhodamine methyl ester (TMRM), 40 nM for 30 min at room temperature. MitoFluor Green (AM ester), 20 nM for 30 min at room temperature. Rhod-2 (AM ester): 5 μM with 0.005% pluronic for 30 min at 37 °C in maintenance medium and under such conditions, the dye became partitioned and hydrolyzed in mitochondria. Cells were washed with control buffer after dye loading.

2.4. Confocal microscopy

Confocal microscopy was performed using an LSM510 confocal laser scanning microscope (Carl Zeiss). MitoFluor Green was excited using the 488 nm argon laser line and emission collected at 505–550 nm, while TMRM and rhod-2 were excited using the 543 nm HeNe laser line and emitted light collected at >560 nm.

2.5. Interleukin-6 and inhibitor pre-treatment

Cardiomyocytes were treated with 10 ng/ml murine IL-6 for 6 h. Maintenance medium was replaced for a further 18 h prior to experimental procedures. To determine the role of the PI 3-kinase/Akt pathway, cardiomyocytes were pre-treated with 100 nM wortmannin (diluted 1:1000 from a stock solution in DMSO) for 30 min prior to IL-6 administration. Where appropriate, inhibition of iNOS was
achieved by treating cardiomyocytes with 200 µM amino-guanidine for 30 min prior to sI/R, 24 h post-treatment with IL-6.

2.6. Models of simulated ischemia-reperfusion (sI/R)

2.6.1. (a) Simulated ischemia model to assess the degree of IL-6 induced protection

Simulated ischemia (sI) was carried out 24 h after IL-6 pre-treatment. Culture medium was replaced with modified Krebs buffer (as for control buffer but without glucose) and supplemented with 10 mM 2-deoxyglucose, 20 mM sodium lactate, pH 6.8 and hypoxia achieved by placing culture plates into BBL GasPak anaerobic pouches (Becton Dickinson). When oxygen concentration was reduced to below 1% (determined by an indicator strip within pouch), cells were left for 6 h, before ‘reperfusion’. Medium was sampled after 2 h of simulated reperfusion for determination of creatine phosphokinase (CPK) release, as a measure of cell injury [18]. Cell viability was assessed after 24 h of simulated reperfusion, by methylthiazoyltetrazolium (MTT) assay [18].

2.6.2. (b) sI/R setup for fluorescence imaging of cardiomyocytes

For imaging studies it was necessary to use a modified sI/R protocol in order to be compatible with perfusion on the microscope stage. Cardiomyocytes cultured on coverslips were placed in a perfusion chamber on the stage of an inverted epifluorescence microscope, as previously described [19]. Cells were perfused at ~10 ml/min and maintained at 35±2°C. The sI/R protocol entailed 1 h of perfusion with sI buffer (composition as above) equilibrated with argon and supplemented with the oxygen scavenger sodium dithionite (750 µmol/L) immediately prior to perfusion. P02 values of <130 Pa (1 mm Hg) were achieved, as monitored using a carbon fibre electrode. Simulated reperfusion (re-oxygenation) for up to 1 h was performed by perfusion with control buffer, equilibrated with air. This method resulted in approximately the same extent of cell death as the protocol described in (a) because sodium dithionite scavenges oxygen very rapidly compared to the relatively slow onset of hypoxia using the GasPak system.

2.7. Monitoring Changes in [Ca2+] during sI/R

Fura-2 fluorescence was measured throughout sI/R: cells were illuminated using a 100 W xenon arc lamp via a spinning filter wheel (Cairn Research) fitted with appropriate 10 nm bandpass filters at 340, 360 and 380 nm. Illumination was limited to 2 s every minute by use of a shutter, in order to minimise photodynamic damage and emitted light was collected with a PMT using a bandpass filter at >530±15 nm. Data was recorded using a CCD based imaging system.

2.8. Measurement of Ca2+ transients in cardiomyocytes

Using the spinning filter wheel set-up described above, oscillations in [Ca2+]c were measured in Fura-2 loaded cardiomyocytes, maintained at ~35 °C by perfusion with control buffer. Ca2+ transients were sampled for 10 s periods, in at least 42 individual cells, per treatment (Control: n=60; IL-6: n=60; IL-6+ aminoguanidine: n=42). Treatment with amino guanidine (AG: 200 µM) was for 30 min prior to measurement of calcium transients.

2.9. Measurement of mitochondrial membrane potential during reperfusion

In order to identify all mitochondria regardless of polarisation state, cells were pre-loaded with MitoFluor Green (20 nM), prior to simulated ischemia. Then following 1 h of simulated ischemia, cardiomyocytes were reperfused for 5 min with control buffer and loaded with low-dose (40 nM) TMRM for 30 min and imaged by confocal microscopy. At low TMRM concentrations there is a direct relationship between inner membrane potential and TMRM fluorescence. Changes in mitochondrial membrane potential were determined by measuring the ratio of TMRM to mitofluor green signals. A threshold setting was used to exclude pixels from the cytosol and the TMRM signal from remaining pixels over mitochondria (as identified by MFG fluorescence) was taken.

2.10. Assessment of mitochondrial Ca2+ uptake during reperfusion

Cardiomyocytes were pre-loaded with rhod-2 and subjected to 1 h of simulated ischemia. Following a brief 5-minute reperfusion to allow equilibration of buffers, cells were imaged by confocal microscopy during the 60 min of ‘no-flow’ reperfusion. In order to measure cytosolic Ca2+ by confocal fluo-4 was used since fura-2 was not suitable for use with the UV laser and fluo-4 allows simultaneous measurement of the cytosolic and mitochondrial (rhod-2) signals.

2.11. Immunoblotting

Cardiomyocytes were harvested in 200 µl per well of 2× SDS-PAGE sample buffer and heated to 100 °C for 5 min followed by the addition of 5 µl of bromophenol blue (8% in ethanol). Samples were separated at 85 V on 10% SDS-PAGE gels using mini-gel apparatus (Biorad, UK) and then blotted onto nitrocellulose membranes (Hybond-C, Amersham, UK) overnight at 35 mA using a mini-transblot apparatus (Biorad, UK). Blots were developed as previously described [18]. Primary antibodies were as described in materials and in the figure legends and secondary antibody was peroxidase-conjugated swine anti-rabbit IgG (DAKO, UK).
3. Results

3.1. IL-6 confers NO-dependent delayed protection of cardiomyocytes in cellular models of sI/R

The ability of IL-6 pre-treatment to protect neonatal rat cardiomyocytes was assessed using a simulated ischemia/reperfusion (sI/R) model of cell injury. As shown in Fig. 1A, sI/R resulted in approximately 54% cell survival as determined by MTT assay. However, pre-treatment of cardiomyocytes with IL-6 resulted in a significant increase in cell viability to approximately 70% of control (p < 0.0001). This increase in survival was accompanied by a greater preservation of sarcolemmal integrity as measured by CPK release (CPK release was approximately 54% of that associated with sI/R; p < 0.001) (Fig. 1B).

IL-6 pre-treatment was associated with the elevation of iNOS activity. Therefore, we also determined whether the protection against sI/R injury induced by IL-6 was NO dependent. To test this, cardiomyocytes were pre-treated with 10 ng/ml IL-6 for 6 h and then recovered in normal maintenance medium for a further 18 h. The cells were then treated with the selective iNOS inhibitor amino guanidine (AG) for 30 min prior to the sI/R protocol. AG treatment abrogated the protective effect of IL-6 as determined by both cell viability (MTT bioreduction: Fig. 1A) and cell injury (CPK release: Fig. 2B). However, AG alone had no effect on survival in the absence of IL-6, suggesting that it is the iNOS-derived NO that confers protection immediately prior to and during sI/R.

3.2. IL-6 reduces cytosolic calcium overload during simulated ischemia-reperfusion

We next determined changes in cytosolic calcium concentration ([Ca^{2+}]_c) during sI/R as described in the methods section. As shown in Fig. 1C, simulated ischemia induced a gradual increase in the fura-2 ratio ([Ca^{2+}]_c) over 60 min, which returned rapidly to baseline upon reperfusion. Oscillations in [Ca^{2+}]_c and spontaneous beating returned briefly before a subsequent secondary, exponential increase in [Ca^{2+}]_c ensued, followed by the loss of membrane integrity and of the fura-2 signal. In contrast, IL-6 pre-treatment significantly reduced the magnitude of the [Ca^{2+}]_c rise during ischemia, from 315 ± 69% in untreated controls to 147 ± 36% in IL-6 pre-treated cells (n=8, p<0.0001). Furthermore, IL-6 pre-treatment suppressed the secondary increase in [Ca^{2+}]_c (no significant increase, compared with 724 ± 127%, in untreated cells, before cell rupture). In these cells, membrane integrity, spontaneous beating and accompanying [Ca^{2+}]_c oscillations were maintained until the end of the experiment (data not shown).

3.3. IL-6-induced iNOS expression and delayed protection are PI 3-kinase-dependent

IL-6 activates a number of signalling pathways in cardiomyocytes including JAK/STAT, Erk1/2, p38-MAPK and PI 3-kinase/Akt. Since our previous data has suggested a protective role for PI 3-kinase, we next determined the role of the PI 3-kinase/Akt pathway in IL-6-dependent delayed protection. Isolated cardiomyocytes were pre-treated with vehicle or the PI 3-kinase inhibitor wortmannin prior to IL-6 treatment and cells were harvested after 60 min for analysis of Akt/protein kinase B (PKB) phosphorylation. As shown in Fig. 2A (i), PKB Ser^{473} phosphorylation was significantly increased at 60 min in response to IL-6 and this was prevented by wortmannin. IL-6 pre-treatment also resulted
in a significant and time-dependent increase in the expression of the inducible isof orm of NO synthase (iNOS; NOS2) [Fig. 2A (ii)]. iNOS expression was also abrogated by wortmannin, suggesting that iNOS expression was PI 3-kinase-dependent. We did not detect any changes in the expression of constitutive eNOS (NOS3) or nNOS isoforms in response to IL-6 (not shown), suggesting that this effect was specific to iNOS. In addition, the protection afforded by IL-6 pre-treatment was completely abolished by wortmannin as determined by measurements of MTT bioreduction and CPK release (Fig. 2B). Therefore, in cardiomyocytes, IL-6-dependent cytoprotection is mediated by a PI 3-kinase and iNOS-dependent mechanism.

3.4. IL-6 reduces the amplitude of \([Ca^{2+}]_c\) oscillations – an effect dependent upon NOS

Isolated neonatal cardiomyocytes display spontaneous \([Ca^{2+}]_c\) transients that accompany their rapid contraction [as shown in Fig. 3, panels (i) to (iii)]. Since we observed that IL-6 pre-treatment resulted in a reduction in \([Ca^{2+}]_c\) during sl/R, we next examined the effect of IL-6 pre-treatment on \([Ca^{2+}]_c\) oscillations. Representative changes in the fura-2 ratio in control cells is shown in panel (i). The mean amplitude of oscillations in control cardiomyocytes was found to be 1.132 (arbitrary units) as shown in Fig. 3 [panel (iv)]. Pre-treatment with IL-6 caused a significant decrease in the amplitude (peak ratio) of spontaneous waves as shown in panel (ii) with a mean of 0.642 (57%, \(p < 0.0001\)) [panel (iv)]. There was no obvious change in the width of the Ca2+ response (measured at half maximum) or in the frequency of the spontaneous waves.

In view of the observed IL-6-dependent up-regulation of iNOS levels in neonatal rat cardiomyocytes, we examined the potential role of NO in mediating the IL-6 induced \([Ca^{2+}]_c\) transient reduction. A 30 min pre-treatment with the selective iNOS inhibitor amino guanidine (AG) blocked the reduction in Ca2+ transient amplitude induced by IL-6 [panel (iii)], producing a transient with a modestly greater amplitude compared with control cells [not significant vs. control: panel (iv)]. Treatment of cells with AG alone did not significantly affect \([Ca^{2+}]_c\) transients (data not shown).

3.5. IL-6-induced modulation of mitochondrial function

To test whether the IL-6 mediated effects were associated with changes in mitochondrial function, we next examined the effects of IL-6 on mitochondrial energetics. Control and IL-6 pre-treated cells were loaded with TMRM or rhod-2 and imaged by confocal microscopy in order to study the effects of the cytokine on mitochondrial membrane potential and mitochondrial Ca2+ (rhod-2 fluorescence) respectively. To ensure the localisation of TMRM and rhod-2 to mitochondria cells were also loaded with mitofluor green (MFG). Both TMRM and MFG strongly localised to mitochondria (Fig. 4 A). Following IL-6 pre-treatment TMRM and rhod-2 showed a similar distribution (Fig. 4B). Interestingly, IL-6 pre-treatment resulted in a small, but reproducible and significant increase in the mean TMRM signal intensity indicating an increase in the inner mitochondrial membrane (IMM) polarisation (i.e. \(\Delta \Psi_m\) becomes more negative) (Fig. 4 C). In parallel we observed that basal mitochondrial Ca2+ (rhod-2 fluorescence) was also significantly elevated in IL-6 pre-treated cells (Fig. 4D). To confirm that the IL-6-induced increase in rhod-2 signal represented a genuine increase in
[Ca\(^{2+}\)]_m and not simply due to an increase in TMRM and rhod-2 loading, the mitochondrial uncoupler FCCP was applied to cells co-loaded with the cytosolic calcium indicator fluo-3 in order to dissipate the potential. Mitochondrial rhod-2 fluorescence decreased, with a concomitant rise in cytosolic calcium, showing that collapse of mitochondrial potential causes a loss of mitochondrial calcium to the cytosol (Fig. 4E). Therefore, IL-6 treatment results in a modest increase in IMM polarisation and mitochondrial Ca\(^{2+}\) loading.

### 3.6. Effect of sI/R on mitochondrial function

Because TMRM is a potentiometric dye, the TMRM signal is lost when mitochondria depolarise in response to sI/R. In order to overcome this problem, cardiomyocytes were pre-loaded with Mitofluor Green (MFG), prior to sI/R. The MFG signal intensity and distribution were shown to be relatively unaffected by sI/R (Fig. 5). Following sI/R, cardiomyocytes were loaded with low-dose TMRM and imaged by confocal microscopy after 30 min “no-flow reperfusion” (time at completion of dye loading). Whilst the MFG signal appears to be uniform in intensity throughout the mitochondrial population, that of TMRM appeared to be greater in perinuclear mitochondria, possibly reflecting differences in \(\Delta\psi_m\) energy status in different populations of mitochondria within the same cell. Furthermore, at the earliest time of imaging post-reperfusion (300 R), \(\Delta\psi_m\) was already more negative in some cells, as indicated by a higher TMRM signal, compared with control cells (as shown in Fig. 5A and B). \(\Delta\psi_m\) increased further in most cells (82%) through 40–50 min reperfusion (Fig. 5B), becoming maximally polarised after approximately 60 min (Fig. 5A and B). This approach of imaging the mitochondria therefore provided a reliable method of assessing the changes that occur during the sI/R protocol.

Interestingly, depolarisation occurred earlier in mitochondria at the outer extremities of the cell, while perinuclear mitochondria retained membrane potential for up to 70 min (Fig. 6A). Depolarisation later ensued in this population of mitochondria so that they were completely depolarised by 80 min (Fig. 5A and B). As such, all mitochondria imaged through 80–105 min were markedly depolarised, having little, if any, TMRM signal (Figs. 5B and 6B). However, after 110 min of reperfusion, two distinct populations of
cardiomyocytes became apparent: firstly, those in which mitochondria remained structurally intact, which repolarised and resumed spontaneous beating and in which the cells then remained viable for the duration of the experiment. This was observed in 37.2% of cells (see for example Figs. 5A, B, and 6C). In contrast, in 62.8% of cells the mitochondria failed to repolarise and large-amplitude mitochondrial swelling was observed in many cells as early as 80 min following reperfusion (for example, see Fig. 5A, 80'R, Figs. 6B and 7, 60'R control), before cell rupture occurred and dyes were lost. The survival rate of 37.2%, determined here by imaging, agrees well with the values derived from our viability (MTT) assays (36%). It was noted that cells in which mitochondria were more polarised prior to Δψ_m collapse were less likely to repolarise and were more susceptible to lysis (data not shown).

3.7. IL-6-induced modulation of mitochondrial energetics during sI/R

Having characterized the changes in mitochondrial membrane potential during reperfusion in control cells,

![Figure 4](image_url)

**Fig. 4.** Δψ_m and [Ca^{2+}]_m are increased in IL-6 pre-treated cardiomyocytes under resting conditions. Cardiomyocytes, loaded with 40 nM TMRM, 20 nM MitoFluor Green (MFG) or both dyes in combination, imaged by confocal microscopy during sI/R. The TMRM and MFG signals co-localise to mitochondria in cardiomyocytes (A). Confocal images of cells loaded with either 40 nM TMRM or 5 μM Rhod-2 (B) to illustrate localisation of these dyes to mitochondria following IL-6 pre-treatment. IL-6 induced a modest increase in Δψ_m (C) and a significant rise in [Ca^{2+}]_m (D), compared with control cells. ****: p<0.0001. Ca^{2+} (Rhod-2) signal was lost when cells were ‘puffed’ with the mitochondrial uncoupler FCCP (1 μM) to depolarise the mitochondria (E). Panel B shows different cells, since it was not possible to image TMRM and Rhod2 simultaneously in the same cell.
we examined whether any of these parameters were affected by IL-6 pre-treatment, with a view to understanding the mechanism of protection. Following simulated ischemia, cells were briefly reperfused, loaded with 40 nM TMRM and imaged by confocal microscopy following 30–60 min ‘no-flow reperfusion’. The mitochondrial membrane potential increased above control level following sI/R (314%, p < 0.0001, see Fig. 6D-post sI/R and E), in keeping with the hyperpolarisation observed in MFG co-loaded cells. However, pre-treatment with IL-6 resulted in a decreased post-sI/R hyperpolarisation (167% of control D_w_m, p < 0.001 vs. sI/R, see Fig. 6D and E). Since the degree of hyperpolarisation correlated with the likelihood of cell death, this correlates with the reduced cell death following IL-6 pre-treatment. In agreement with this, mitochondrial morphology was noticeably disrupted following sI/R in control cells (Fig. 6B), whilst structural integrity was largely preserved in the majority of IL-6 pre-treated cells. In an attempt to quantify the degree of morphological disruption, mitochondrial form was studied by particle size analysis. The size of particles (mitochondria) identified using TMRM fluorescence was determined to be significantly increased following sI/R (approximately 3-fold, p < 0.001, see Fig. 6B and F) indicating clumping and swelling of mitochondria. In contrast mitochondrial structural integrity was largely conserved by IL-6 pre-treatment and particle size analysis showed that mitochondrial morphology was indistinguishable from control (Fig. 6D and F).

3.8. IL-6-induced modulation of mitochondrial Ca^{2+} uptake during sI/R

In order to determine whether the hyperpolarisation during reperfusion was also driving increased mitochondrial Ca^{2+} uptake, cells were pre-loaded with rhod-2 AM prior to simulated ischemia, reperfused for 10 min and then imaged by confocal microscopy. Because the rhod-2 can be pre-loaded into cells (in contrast to TMRM, where this was not possible) we were able to observe changes in [Ca^{2+}]_m at earlier time points during reperfusion than was possible for TMRM. The results show that in control cells [Ca^{2+}]_m increased considerably during reperfusion, in parallel with Δψ_m (Fig. 7). Although [Ca^{2+}]_m was initially higher at baseline in IL-6 pre-treated cells, after 60 min of reperfusion loss of mitochondrial Ca^{2+} was observed in control cells but not IL-6 pre-treated cells. This correlated with the difference in cell survival observed at these later time points. This time course of events was consistent with the contracture and loss of cell-matrix attachments at 60 min of reperfusion observed in control MTG-loaded cells (Fig. 5B). Hyperpolarisation preceded Δψ_m collapse and appeared to be a predictor of this event. In control cells
Fig. 5. Mitochondrial hyperpolarisation precedes depolarisation during simulated reperfusion. Following simulated ischemia, MFG pre-loaded cells were reperfused, loaded with 40 nM TMRM and imaged by confocal microscopy over 30–120 min (A). Mitochondrial hyperpolarisation was observed in 82% of cardiomyocytes after 50–70 min reperfusion (as shown upper right at 60’R). This was followed by a depolarisation in all cells imaged during the 80–100 min time interval (as shown in the lower panels at 80’ through 110’R). 62.8% of cardiomyocytes remained depolarised, many of which displayed mitochondrial swelling and ultimately cell lysis. However, in 37.2% of cells, mitochondria repolarised, spontaneous beating resumed and viability was preserved. (B–D) Ratios of TMRM: MFG were calculated for individual mitochondria and an average, per cell, is represented by each point (B–D). The dotted line indicates the average TMRM:MFG ratio of control cells, to enable interpretation of relative hyperpolarisation and depolarisation.
Fig. 6. IL-6 pre-treatment limits mitochondrial hyperpolarisation and leads to preservation of mitochondrial integrity. Cardiomyocytes were pre-loaded with mitofluor green and then following simulated ischaemia, cardiomyocytes were loaded with 40 nM TMRM in control buffer for 30 min, prior to confocal imaging (A–C) or with TMRM alone (D–F). Mitochondria at the periphery of the cells depolarised earlier than perinuclear mitochondria (A). Morphological disruption following sI/R was associated with large amplitude swelling (B). Surviving cardiomyocytes showed mitochondrial re-polarisation and maintenance of structural integrity (C). Simulated ischaemia and 30–60' reperfusion produced an increase in ΔΨm (D, E), but this was significantly attenuated by pre-treatment with IL-6. Mitochondrial morphology was severely disrupted following sI/R, by direct observation and as determined by particle size analysis (F). As a result of IL-6 pre-treatment, cardiomyocytes retained cellular integrity and intact mitochondrial morphology (C, D and F). **: p<0.001 vs. control; ***: p<0.0001 vs. control; † †: p<0.001 vs. control; † † †: p<0.0001 vs. sI/R.
mitochondria underwent large-amplitude swelling prior to loss of mitochondrial Ca\(^{2+}\) to the cytosol (for example Figs. 5A 80\(^R\) and 6B). These cells underwent only a brief period of spontaneous beating before cell rupture occurred. In contrast, mitochondria from IL-6 pre-treated cells remained viable and [Ca\(^{2+}\)]\(_{m}\) remained unchanged. Furthermore, in IL-6 pre-treated cardiomyocytes the [Ca\(^{2+}\)]\(_{m}\) returned to basal levels and spontaneous contraction resumed and was maintained for the duration of the experiment. In accordance with previous experiments in this study, considerably more IL-6 pre-treated cells survived after 60 min reperfusion compared with control cells. These results confirm that IL-6 increases the survival of cardiomyocytes by altering IMM potential and controlling mitochondrial Ca\(^{2+}\) loading post-sI/R.

4. Discussion

In this study we have demonstrated that IL-6 induces an increase in iNOS expression and a NO-dependent suppression of both calcium transients and associated contraction in cardiomyocytes. These effects were accompanied by an increase in mitochondrial inner membrane polarisation (\(\Delta\psi_{m}\)) and mitochondrial calcium content in response to IL-6. Furthermore, pre-treatment
of cardiomyocytes with IL-6 completely abrogated the loss of $\Delta \psi_m$ and subsequent secondary cytosolic calcium overload in response to simulated ischaemia-reperfusion and reduced cell death which occurred in a PI 3-kinase and NO-dependent manner. Therefore, in this model, IL-6 induces PI 3-kinase and NO-dependent cytoprotection which was associated with changes in calcium handling. This could have important implications for the targeting of cytokine-mediated pathways for the treatment of post-infarction remodelling and heart failure and also in the delineation of cardioprotective signalling pathways.

$\Delta \psi_m$ induced hyperpolarisation in the majority of mitochondria, peaking at 60 min post-‘reperfusion’ which was followed by mitochondrial swelling, subsequent depolarisation and a loss of cell viability. The degree of initial hyperpolarisation appeared to correlate with the likelihood of subsequent depolarisation and loss of viability. An increase in mitochondrial potential in response to $\text{sI/R}$ which precedes mitochondrial depolarisation and loss of integrity has recently been observed by others [20]. However, both $[\text{Ca}^{2+}]_m$ and $\Delta \psi_m$ were maintained throughout in IL-6 pre-treated cells. Thus it would appear that part of the protective effect of IL-6 is due to an increased capacity of the mitochondria to take up and retain $\text{Ca}^{2+}$ whilst preventing depolarisation.

The study by Beltran and colleagues [21] demonstrated that in the presence of a NO-donor (DETA-NO) at concentrations that inhibit mitochondrial respiration, $\Delta \psi_m$ was maintained in the face of serum deprivation and that the cells were protected against apoptosis. In this case, $\Delta \psi_m$ was maintained by the activity of electrogenic pumps such as the reversed ATP synthase and the adenine nucleotide translocator (ANT) and was dependent on the consumption of glycolytic ATP. Since the effects on cardiomyocyte survival that we observed were also NO-dependent, it is possible that a similar mechanism occurs in response to IL-6. IL-6 and related cytokines have also been shown to stimulate glycolysis [22], which would facilitate this mechanism.

Protective versus injurious effects of NO are critically dependent on the concentration and duration of NO exposure and the relative reversibility of inhibition of mitochondrial respiration. For instance, the transient reversible inhibition of complex IV cytochrome oxidase by NO due to competition with $O_2$ appears to be protective. In contrast, prolonged exposure to high levels of NO leads to inhibition of complex I due to covalent nitrosylation which may precipitate the mitochondrial permeability transition (MPT) and cause the collapse of $\Delta \psi_m$ [23–25]. NO-dependent mitochondrial hyperpolarisation has been reported to occur in T-cells [26,27].

Thus a NO-dependent increase in $\Delta \psi_m$ such as we observed following IL-6 pre-treatment in cardiomyocytes is distinct from the reoxygenation-induced hyperpolarisation and appears to represent a common mechanism of regulation of mitochondrial energetics and cell fate decisions. Transient mitochondrial uncoupling appears to be a component which underlies several forms of acute cardioprotection such as ischaemic preconditioning [28] and the opening of mitochondrial ATP-sensitive potassium channels (mito-K$_{\text{ATP}}$) [29] which prevent or delay the formation of MPT and therefore may represent a general protective mechanism. Furthermore, since we observed that both iNOS induction and protection in response to IL-6 were blocked by wortmannin, PI 3-K also represents a major pathway involved in delayed protection in response to IL-6 and agrees with previous reports that the PI 3-kinase/Akt pathway mediates cardioprotection [30].

The accumulation of intramitochondrial $\text{Ca}^{2+}$ plays an important role in the regulation of subcellular calcium homeostasis [31] and also in the delineation of cardioprotective signalling pathways. Mitochondria are the targets for the targeting of cytokine-mediated pathways for the treatment of post-infarction remodelling and heart failure and also in the delineation of cardioprotective signalling pathways.

In conclusion, it is clear from these studies that IL-6 pre-treatment induces a delayed cytoprotective response which is dependent on PI 3-kinase and NO and causes changes in...
mitochondrial function that are associated with an increase in potential and an increased ability of mitochondria to accumulate intracellular Ca\textsuperscript{2+} without undergoing terminal depolarisation.

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