Mitochondrial oxidant stress triggers cell death in simulated ischemia–reperfusion ☆

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ABSTRACT

To clarify the relationship between reactive oxygen species (ROS) and cell death during ischemia–reperfusion (I/R), we studied cell death mechanisms in a cellular model of I/R. Oxidant stress during simulated ischemia was detected in the mitochondrial matrix using mito-roGFP, a ratiometric redox sensor, and by Mito-Sox Red oxidation. Reperfusion-induced death was attenuated by over-expression of Mn-superoxide dismutase (Mn-SOD) or mitochondrial phospholipid hydroperoxide glutathione peroxidase (mito-PHGPx), but not by catalase, mitochondria-targeted catalase, or Cu,Zn-SOD. Protection was also conferred by chemically distinct antioxidant compounds, and mito-roGFP oxidation was attenuated by NAC, or by scavenging of residual O_2 during the ischemia (anoxic ischemia). Mitochondrial permeability transition pore (mPTP) oscillation/ opening was monitored by real-time imaging of mitochondrial calcein fluorescence. Oxidant stress caused release of calcein to the cytosol during ischemia, a response that was inhibited by chemically diverse antioxidants, anoxia, or over-expression of Mn-SOD or mito-PHGPx. These findings suggest that mitochondrial oxidant stress causes oscillation of the mPTP prior to reperfusion. Cytochrome c release from mitochondria to the cytosol was not detected until after reperfusion, and was inhibited by anoxic ischemia or antioxidant administration during ischemia. Although DNA fragmentation was detected after I/R, no evidence of Bax activation was detected. Over-expression of the anti-apoptotic protein Bcl-X_L in cardiomyocytes did not confer protection against I/R-induced cell death. Moreover, murine embryonic fibroblasts with genetic depletion of Bax and Bak, or over-expression of Bcl-X_L, failed to show protection against I/R. These findings indicate that mitochondrial ROS during ischemia triggers mPTP activation, mitochondrial depolarization, and cell death during reperfusion through a Bax/Bak-independent cell death pathway. Therefore, mitochondrial apoptosis appears to represent a redundant death pathway in this model of simulated I/R. This article is part of a Special Issue entitled: Mitochondria and Cardioprotection.

1. Introduction

Tissue ischemia is characterized by severe hypoxia, acidosis, energy depletion and cell death. Although timely restoration of blood flow is currently the most effective means of minimizing ischemic injury, reperfusion of ischemic tissue triggers a paradoxical increase in cell death [6]. Excessive oxidant stress is well accepted as an important component of ischemia–reperfusion (I/R) injury [5,6]. ROS production begins early in ischemia, and is followed by a large burst of oxidant stress during the first few minutes of reperfusion [2,30,41,46]. Potential sources of ROS include mitochondria, NAD(P)H oxidases, nitric oxide (NO) synthase, and xanthine oxidase, and the critical targets of oxidant stress may include proteins, membrane lipids, and DNA [7,14,25,37]. Although many of the details regarding the sources and targets of oxidant stress during I/R are not known, a consensus regarding the importance of ROS in I/R injury has developed, based on studies showing that cells are protected during I/R by pretreatment with antioxidants or by over-expression of antioxidant enzymes [8,9,16,34].

Other reports have implicated mitochondrial apoptosis in the cell death triggered by I/R. This highly conserved process can be initiated by the activation of BH3-containing pro-death proteins in response to a variety of stimuli including hypoxia, nutrient deprivation, ROS and DNA damage [20]. Apoptotic triggers cause the Bcl-2 family members Bax and Bak to translocate to the mitochondria and trigger the release of cytochrome c to the cytosol [19]. This leads to the activation of caspases and subsequent cell death through an ATP-dependent pathway [42,49]. Consistent with that model, transgenic mice that

over-express Bcl-2, a Bax/Bak suppressor, or that are deficient in the Bax protein, have been reported to exhibit smaller infarcts compared with wild-type mice subjected to I/R [23,26,43]. Other studies report that pharmacological inhibition of caspases is protective against I/R injury in vitro [36,40] although there is debate as to whether caspase inhibition can prevent cell death following cytochrome c release to the cytosol, due to the existence of redundant effector pathways of apoptosis that act downstream of the mitochondria.

Although oxidant stress and apoptosis have both been implicated in I/R-induced cell death, the relationship between these processes is not clearly established. One possibility is that ROS generation during I/R leads to the BH3-dependent activation of mitochondrial apoptosis, cytochrome c release, and caspase-mediated cell death. Alternatively, oxidant stress generated during I/R could trigger opening of the mitochondrial permeability transition pore, leading to cytochrome c release to the cytosol, bioenergetic failure, and cell death by a necrotic rather than an apoptotic pathway [11,12]. In the former case cytochrome c (cyt-c) release would mediate cell death, whereas in the latter case the release of cyt-c would represent only a marker of lethal cell damage. The present study examined the relationship between oxidant stress during I/R and mitochondria-mediated cell death, using a model of simulated I/R in which the cells were superfused with hypoxic, hypercarbic and acidotic medium lacking glucose. The data show that oxidant stress generated during simulated ischemia triggers oscillation of the mitochondrial permeability transition pore, and that this response precedes the irreversible opening of the pore and cyt-c release during reperfusion. Although cytochrome c release during reperfusion leads to caspase activation, interventions that inhibit BH3-dependent apoptosis do not protect against cell death in this model. These findings indicate that apoptosis becomes activated in simulated I/R, but it is not required for subsequent cell death.

2. Methods

2.1. Cell culture and perfusion system

Embryonic chick cardiomyocytes were prepared as previously described and grown at a density of 1×10^5 on 25 mm glass cover slips or 6 well plates in a humidified incubator [47]. Experiments were performed on spontaneously contracting cells at 3–5 days after isolation. Primary mouse embryonic fibroblasts (MEFs) derived from wild type and Bax-Bak double knockout mice were immortalized with a plasmid containing SV40 genomic DNA. Immortalized wild-type MEFs were also used to develop a stable cell line over-expressing Bcl-X_L. MEFs were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (200 mmol/L) and antibiotics (pen/strep). Embryonic stem cells devoid of cytochrome c (cyt-c$^{-/-}$ ES cells) were cultured under conditions that compensate for their defect in mitochondrial respiration using media supplemented with uridine and pyruvate as previously described [31]. Wild-type embryonic stem cells containing cytochrome c were cultured in parallel with null (cyt-c$^{-/-}$) ES cells and used as controls.

2.2. Simulated ischemia–reperfusion

Simulated I/R was achieved in a flow through chamber as previously described [47]. The chamber was comprised of two cover slips separated by a stainless steel spacer ring, with the cultured cells on the lower cover slip. The space between the cover slips was perfused with balanced salt solutions (BSS) at a flow rate of 0.5 mL/min. All solutions were equilibrated by bubbling vigorously with calibrated gases in a heated, water jacketed reservoir (Radnoti Glass Co.). The reservoir was connected to the flow-through chamber with stainless steel tubing to minimize entry of ambient oxygen. The chamber was mounted on a heated stage (37 °C) on an inverted

microscope (Nikon). During baseline conditions the cells were superfused for 20 min with BSS (NaCl 117 mmol/L, KCl 4 mmol/L, NaHCO$_3$ 18 mmol/L, MgSO$_4$ 0.76 mmol/L, NaH$_2$PO$_4$ 1 mmol/L, CaCl$_2$ 1.21 mmol/L and glucose 5.6 mmol/L) bubbled with a normoxic gas mixture (5% CO$_2$/21% O$_2$/74% N$_2$). The same solution was used during simulated reperfusion. Simulated ischemia was achieved by perfusing the chamber with ischemic media (NaCl 108.9 mmol/L, KCl 8 mmol/L, NaHCO$_3$ 21.4 mmol/L, MgSO$_4$ 0.76 mmol/L, NaH$_2$PO$_4$ 1 mmol/L, CaCl$_2$ 1.21 mmol/L) devoid of glucose and bubbled with 20% CO$_2$/0% O$_2$/80% N$_2$ (pH 6.8). This resulted in an extracellular PO$_2$ of approximately 7 mm Hg within 10 min of hypoxia as determined by a porphyrin-based phosphorescence quenching assay [33].

2.3. Cell viability

Cell death was assessed using propidium iodide (PI, 5 μmol/L) in the perfusate. This dye is excluded from live cells, but enters upon disruption of the plasma membrane. Fluorescence images were obtained using a 10× objective (excitation: 555 nm, emission: 605 nm) at baseline, at the end of ischemia, and at 1, 2 and 3 h into reperfusion. Following the 3 h measurement, cells were permeabilized with digitonin (300 mmol/L) for 45 min. Cell death during the experiment was calculated as the percent of the post-digitonin value. In most experiments, cell death was assessed in 3 separate regions per cover slip using a computer-controlled motorized stage that returned to specific X–Y positions for repeated measurements. An LDH cytotoxicity assay (BioVision) was used to assess cell viability in response to staurosporine treatment and serum withdrawal. LDH is released into the extracellular space upon loss of plasma membrane integrity. The LDH assay was used according to the manufacturer's specifications with minor modifications. Supernatants were collected after treatment with staurosporine (1 μmol/L in 1.5 mL of serum-free media) or prolonged serum withdrawal. Cells were incubated in Triton-X (1.5 mL of 2% in serum-free media) for 30 min. Lysates were collected and centrifuged at 11,000 RPM for 10 min. Samples were mixed with the reaction mixture and incubated for 30 min in a 96 well plate, at which point the absorbance was measured at 490 nm. Cytotoxicity was calculated as the absorbance in the supernatant divided by the sum of absorbance in the supernatant and lysates.

2.4. Mitochondrial membrane potential

$\Delta\Psi$m was assessed with tetramethylrhodamine ethyl ester (TMRE) under non-quenching conditions as previously described [30]. Cells were loaded with TMRE (100 nmol/L) at 37 °C for 30 min. The media were then replaced with a maintenance concentration of TMRE (25 nmol/L). Mitochondrial fluorescence intensity was assessed using excitation at 555 nm and emission at 605 nm. Images were obtained during baseline and at periodic intervals thereafter (10× objective). Intensity values were corrected for background. Stage positions were programmed using data acquisition software (Metamorph, Universal Imaging) allowing repeated images to be obtained from the same fields of cells throughout each experiment.

2.5. DNA fragmentation assay

Cardiomyocytes (2.1×10^6) were disrupted using lysis buffer, and the DNA was extracted using phenol:chloroform after 2 h of proteinase K digestion at 50 °C. Genomic DNA (1.5 μg per lane) was treated with 100 μg/mL of RNase A and run on a 2.0% agarose gel containing 0.06 mg/mL of ethidium bromide. Apoptotic DNA fragments consist of multimers of 180–200 base pairs and appear as a DNA ladder. DNA of non-apoptotic populations of cells has a high molecular weight and does not migrate far into the gel.

2.6. Immunofluorescence labeling of cytochrome c and Bax

To determine subcellular localization of cytochrome c, both treated and untreated cells were fixed in 3% paraformaldehyde with 0.02% glutaraldehyde in PBS for 15 min and subsequently permeabilized with ice-cold methanol. Cells were incubated in 0.5 mg/mL sodium borohydride (Sigma-Aldrich) solution, blocked for 30 min in PBS containing 1% goat serum, and incubated overnight at 4 °C with 1:100 dilution of mouse anti-cytochrome c antibody (BD Bioscience) or mouse anti-Bax (BD Bioscience). Cells were then incubated with a fluorescent secondary antibody (Alexa Fluor 488-labeled anti-mouse IgG, (1:100)) for 1 h at room temperature and visualized under confocal fluorescent microscopy using a 40× objective. Cells with a normal mitochondrial localization of cytochrome c exhibit a fluorescence pattern that is punctate in appearance, while cells that have undergone redistribution of cytochrome c to the cytosol exhibit a more diffuse pattern of fluorescence. Conversely, cells with a normal cytosolic localization of Bax exhibit a fluorescence pattern that is diffuse in appearance while cells that have undergone Bax translocation to the mitochondria exhibit a punctate pattern of fluorescence. Diffuse cells were enumerated independently by two blinded investigators who examined three separate visual fields each containing 50–60 cells. Values were expressed as percent of total cells. In separate experiments, cardiomyocytes were transduced for 48 h with an adenovirus expressing a Green Fluorescent Protein mutant (roGFP) targeted to the mitochondrial matrix using the targeting sequence from cytochrome oxidase subunit IV. This targeting results in expression of the protein exclusively in the matrix compartment [48]. Cells were then subjected to I/R. They were then fixed, immunostained for cytochrome c or Bax, and were imaged using a laser scanning confocal microscope (Zeiss, 60×) to assess the extent of co-localization.

2.7. Reagents

Adenoviruses expressing catalase or mitochondria-targeted catalase were obtained from Drs. Bai and Cederbaum [3]. Adenoviruses expressing Cu,Zn-SOD (SOD I) or Mn-SOD (SOD II) were obtained from the Gene Transfer Vector Core Facility at the University of Iowa [50,51]. Viral doses were established in preliminary studies evaluating expression as a function of viral dosage (multiplicity of infection, MOI, data not shown). The effective concentrations of the chemical antioxidants were reported previously [29,30,41,44]. Embryonic stem cells with homozygous deletion of cytochrome c were obtained from ATCC. TMRE, Calcein-AM, and Alexa-Fluor secondary antibodies were obtained from Molecular Probes-Invitrogen. Digitonin was obtained from Aldrich. Other chemicals were obtained from Sigma.

2.8. Statistical analysis

Data were analyzed by ANOVA, and are reported as mean values ± SEM. When a statistically significant effect was detected, individual differences were explored using a Newman–Keuls post hoc analysis. Statistical significance was determined at the 0.05 level.

3. Results

3.1. Cellular response to simulated I/R

Cardiomyocytes on cover slips were subjected to simulated I/R in a flow-through chamber as previously reported [41]. Phase contrast images revealed evidence of cellular swelling, membrane irregularity, and increased PI uptake during reperfusion (Fig. 1A). To clarify the role of oxidant stress in I/R, antioxidant enzymes targeted to specific intracellular compartments were expressed in cultured cardiomyocytes using adenoviral vectors, and examined in terms of their ability to protect against I/R-induced cell death. The adenoviral titers needed for optimal expression of catalase, catalase targeted to the mitochondrial matrix, Cu,Zn-SOD or Mn-SOD targeted to the mitochondrial matrix, were determined at 48 h for each adenovirus by Western blotting (Fig. 1B). Cells transduced with these adenoviruses or an empty viral vector (Y5) were incubated for 48 h prior to simulated I/R. Paired control experiments were performed on the same day in order to minimize the effects of any differences due to cell age or isolation batch. Negligible cell death was present at baseline, and no significant increases in cell death were detected during ischemia prior to reperfusion (Fig. 1C). During 3 h of reperfusion, cell death increased significantly in cells expressing catalase, mitochondrial catalase or Cu, Zn-SOD, whereas cells over-expressing Mn-SOD showed dramatic protection ($9 \pm 3\%$) compared to Y5 transduced controls ($54 \pm 4\%$). These findings indicate that mitochondrial superoxide, which would have been scavenged by Mn-SOD, must have been a critical contributor to the I/R-induced cell death in the cardiomyocytes.

3.2. Protection by antioxidant compounds

Addition of the antioxidant N-acetyl-L-cysteine (NAC), 2-mercaptopropionyl glycine (2-MPG) or reduced glutathione peptide (GSH) to the perfusate throughout baseline and ischemia, but not during reperfusion, resulted in significant protection from I/R-induced cell death (Fig. 2A). Addition of the O_2 scavenger Oxyrase to the ischemia buffer lowers the O_2 tension to anoxic levels, thereby preventing ROS generation during ischemia. As seen previously [41], Oxyrase administration during ischemia confers significant protection against later cell death during reperfusion (Fig. 2A). However, administration of NAC + 2-MPG only during reperfusion did not result in significant protection compared with control cells. Mitochondrial potential ($\Delta\Psi m$), assessed using TMRE fluorescence, decreased significantly during ischemia, and no recovery of TRME fluorescence was detected during reperfusion (Fig. 2B). Similar responses have been reported previously in this model [30].

3.3. Assessment of mitochondrial oxidant stress during ischemia

To assess oxidant stress in the mitochondrial matrix during ischemia, cells were transduced with an adenovirus expressing mito-roGFP, a ratiometric redox-sensitive sensor [15]. This protein was targeted to the mitochondrial matrix by appending the mitochondrial targeting sequence from cytochrome oxidase subunit IV. The specificity of expression was confirmed by co-localization studies comparing the distribution of mito-roGFP fluorescence and cytochrome c immunostaining, by confocal microscopy (Fig. 3A). Cardiomyocytes expressing mito-roGFP exhibited partial oxidation of the sensor under baseline conditions (Fig. 3B). During simulated ischemia, a significant increase in oxidation was detected, which was abrogated by continuous treatment with NAC, or by anoxic ischemia. Mock ischemia, wherein cells were superfused with ischemic buffer equilibrated with 20% CO_2 and 21% O_2 (no hypoxia), was included to control for possible effects arising from the low pH conditions during ischemia. No significant change in the mito-roGFP oxidation state was detected during mock ischemia, confirming that the changes observed during simulated ischemia were not an artifact arising from low pH conditions. The percent oxidation of the mito-roGFP protein during ischemia was determined by calibrating the sensor at the end of the ischemia challenge. This was achieved by perfusing the cells with media containing dithiothreitol (1 mM), followed by t-butyl hydroperoxide (1 mM) in order to fully reduce and fully oxidize the protein, respectively (Fig. 3C). To confirm the generation of oxidant stress in the mitochondrial matrix during simulated ischemia, cells were loaded with Mito-Sox Red, a cationic probe that distributes to the mitochondrial matrix by virtue of the transmembrane potential (Fig. 3D). Oxidation of this probe was detected during simulated
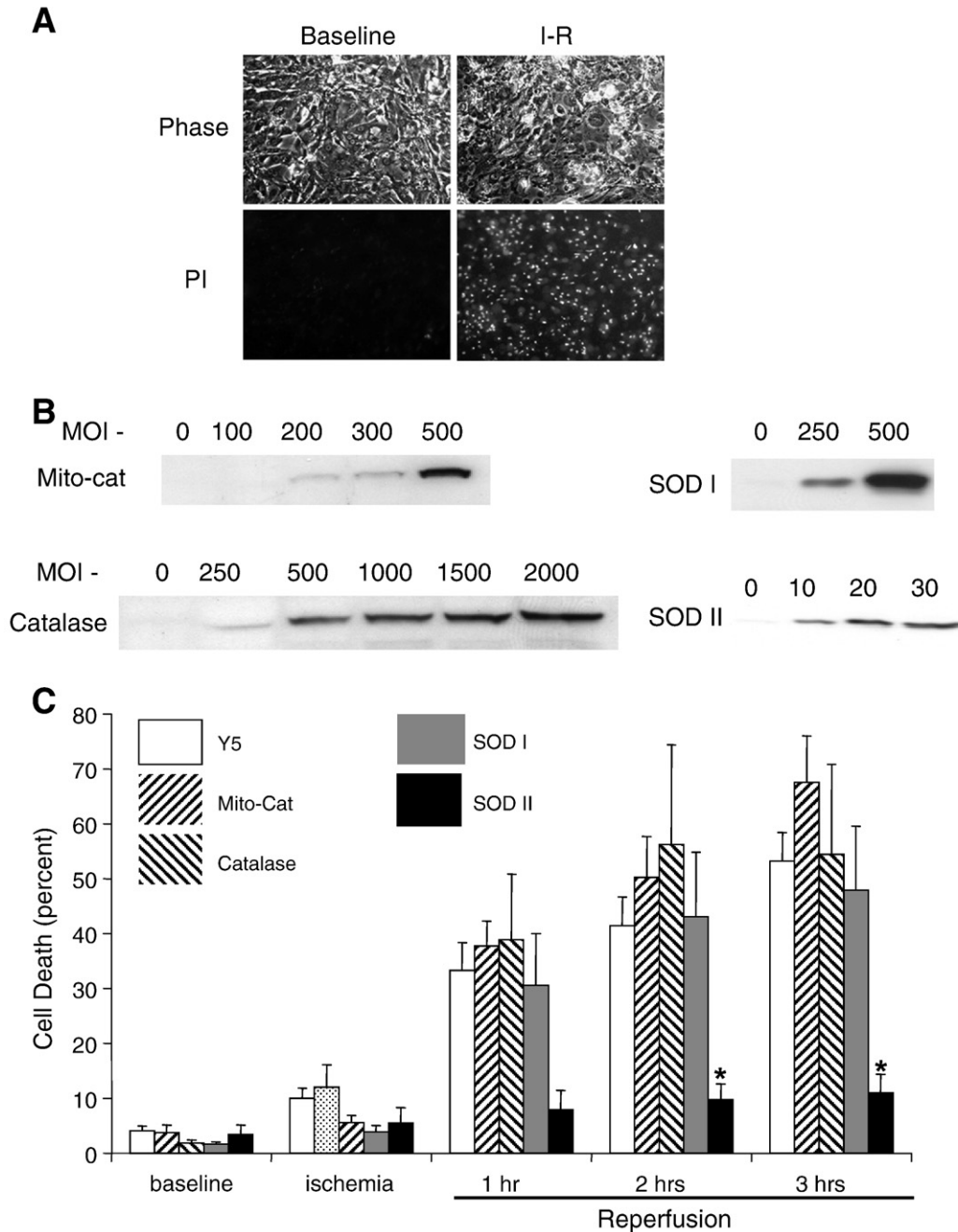
Fig. 1. Cell death in simulated ischemia–reperfusion. (A) Cardiomyocytes exposed to 1 h of simulated ischemia followed by 3 h of reperfusion in a flow-through chamber. Phase images show increased cellular swelling, plasma membrane irregularity and PI uptake after I/R. (B) Western blot analysis of adenovirus-transduced cardiomyocytes showing over-expression of catalase, mitochondria-targeted catalase, Cu,Zn-SOD and Mn-SOD after 48 h. (C) Cardiomyocytes transduced with either Y5 (1000 MOI, n = 10), cytosolic catalase (1000 MOI, n = 6), mitochondria-targeted catalase (500 MOI, n = 6), Cu,Zn-SOD (500 MOI, n = 5) or Mn-SOD (20 MOI, n = 5) for 48 h prior to simulated I/R (*p < .05 compared with controls).

ischemia, but that response was significantly attenuated by NAC administration or by Mn-SOD over-expression (Fig. 3E). Collectively, these findings reveal that simulated ischemia is associated with a significant increase in oxidant stress within the mitochondrial matrix.

3.4. Alterations in mitochondrial morphology induced by ischemic oxidant stress

Cells expressing mito-roGFP exhibited a reticular fluorescence pattern that was concentrated in the perinuclear region. During ischemia this pattern changed, as the fluorescence became more punctate in nature (Fig. 4A). Adult mouse ventricular muscle (400 μm

slice) was harvested, rapidly chilled to prevent ischemic damage, fixed, and examined by transmission electron microscopy. Another slice was subjected to simulated ischemia in the flow-though chamber (30 min), followed by rapid cooling and fixation to permit analysis of the ultrastructural changes associated with ischemia in the absence of reperfusion. In the normal ventricular slice, cardiac muscle fibers in both longitudinal and cross sectional orientations exhibited normal ultrastructure (Fig. 4B, left). Bundles of myofibrils arranged along the length of each muscle fiber were interspersed with numerous mitochondria. Ventricular slices subjected to ischemia without reperfusion showed morphological changes in mitochondria that included rounding and blebbing of the mitochondrial outer
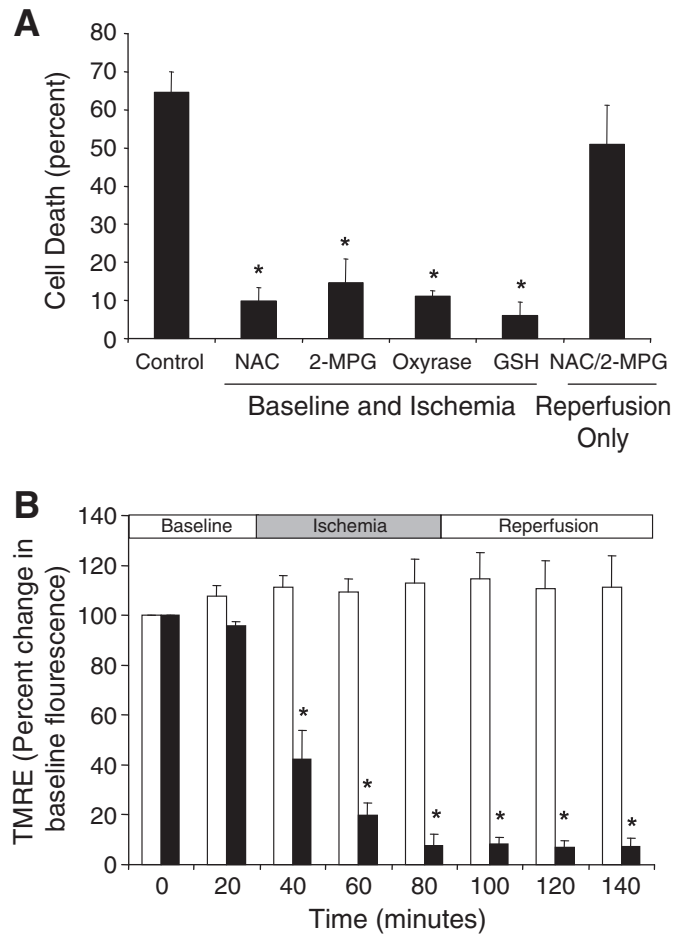
Fig. 2. Oxidant stress and mitochondrial depolarization in simulated ischemia–reperfusion. (A) N-acetyl-ʟ-cysteine (NAC, 500 μmol/L) or 2-MPG (2-mercaptopropionyl glycine, 400 μmol/L) added prior to and during simulated ischemia decreased cell death after 3 h of reperfusion. Similar protection was conferred by anoxic ischemia, achieved by adding Oxyrase to the ischemic media, or by addition of reduced glutathione (GSH, 100 μmol/L) throughout simulated I/R. The combined use of NAC and 2-MPG, beginning 10 min prior to and throughout reperfusion, did not significantly decrease cell death during I/R (Controls, n = 9; NAC, 2-MPG, Oxyrase, n = 4; GSH, n = 3; NAC/2-MPG during reperfusion, n = 4; *p < .05). (B) TMRE fluorescence in cardiomyocytes, to assess changes in mitochondrial potential. Fluorescence intensities were monitored in control cells maintained under normoxia and in cells challenged with simulated I/R. A significant decrease in TMRE fluorescence was detected during 20–40 min of simulated ischemia. Values are expressed as percent of baseline intensity (Controls, n = 6; I/R, n = 4; *p < .05). White bars indicate controls; dark bars indicate I/R.

membrane (Fig. 4B, right). Mitochondria were disorganized and dispersed, in comparison to the tightly packed organization in control slices.

To determine the role of ischemic oxidant stress in the alteration of mitochondrial morphology, the fraction of cells exhibiting fragmentation in the fluorescent labeling studies (Fig. 4A) was quantified (Fig. 4C). Administration of NAC during ischemia, or scavenging of residual O_2 during ischemia with Oxyrase (anoxic ischemia), significantly attenuated the fragmented mitochondrial appearance. These findings implicate ischemic oxidant stress as the cause of these changes in mitochondrial morphology.

3.5. Role of ischemic oxidant stress in regulating the mitochondrial permeability transition pore

Opening of the mitochondrial permeability transition pore (mPTP) at reperfusion has been implicated in causing cyt-c redistribution to the cytosol during I/R [4,11,27]. To determine whether activation of the pore is responsible for the depolarization during ischemia,

mitochondria were loaded with calcein-AM as previously described [38]. After quenching the cytosolic fluorescence with cobalt chloride, a pattern of mitochondrial fluorescence was observed in the cells (Fig. 5A), that remained stable during baseline. During ischemia, the mitochondrial fluorescence pattern shifted to a diffuse cytosolic distribution, which was associated with a significant increase in cellular fluorescence intensity (Fig. 5A, B), indicating escape of calcein from the mitochondrial matrix to the cytosol. Addition of NAC or 2-MPG during baseline and ischemia abolished this response, as did anoxic ischemia achieved using Oxyrase to scavenge O_2 from the ischemia buffer. These findings implicate ischemic oxidant stress in the activation of the mPTP during simulated ischemia.

To further explore the role of mitochondrial oxidant stress in the activation of the mPTP, cardiomyocytes were transduced with adenoviruses to express mitochondria-targeted catalase, Mn-SOD, or mitochondrial phospholipid hydroperoxide glutathione peroxidase (mito-PHGPx). The increase in cytosolic calcein fluorescence during ischemia was abrogated by mito-PHGPx and by Mn-SOD expression, but not by mito-catalase or by infection with the empty Y5 virus (Fig. 5C). Expression of mito-PHGPx was confirmed by Western blotting. Also, mito-PHGPx over-expression conferred protection against cell death in this model (Fig. 5D).

3.6. Relationship between ischemia and reperfusion in terms of cytochrome c release during I/R

The release of cytochrome c from the intermembrane space to the cytosol represents a threshold event in the activation of mitochondrial apoptosis. Cardiomyocytes immunostained for cytochrome c under baseline conditions demonstrated a punctate pattern of fluorescence in confocal images, which exhibited significant co-localization with mitochondria-targeted roGFP, consistent with mitochondrial localization (Fig. 6A). During simulated ischemia, no significant increase in cells exhibiting diffuse cyt-c staining was detected (Fig. 6B). However, a significant increase in the number of cells exhibiting a diffuse pattern of fluorescence was detected during reperfusion, which was mirrored in the cells treated with the apoptosis-inducing agent staurosporine. Treatment of cardiomyocytes with NAC during baseline and ischemia, or with Oxyrase to scavenge O_2 during ischemia, resulted in a significant decrease in the extent of cyt-c redistribution during reperfusion. These findings indicate that, despite release of calcein during the ischemia phase, cyt-c redistribution does not occur until after reperfusion. Moreover, ischemic oxidant stress is required for both the release of calcein during ischemia, and the release of cyt-c during reperfusion in this model.

3.7. Role of mitochondrial apoptosis in simulated I/R

To clarify the requirement for apoptosis in the response to I/R, we examined the role of BH domain-containing proteins in the regulation of cell death. Cardiomyocytes were transduced with an adenovirus to induce over-expression of the antiapoptotic protein Bcl-X_L. While Bcl-X_L over-expression conferred significant protection against cell death induced by staurosporine (data not shown), it did not confer protection against simulated I/R (Fig. 7A). Simulated I/R was not associated with a translocation of Bax from the cytosol to the mitochondria, as evidenced by a lack of co-localization of mitochondria-targeted roGFP and activated Bax (Fig. 7B). Similarly, there was no increase in the percentage of total cells exhibiting a punctate pattern of Bax immunostaining in I/R (Fig. 7C), indicating an absence of Bax oligomerization. However, DNA harvested from cardiomyocytes subjected to I/R did show evidence of laddering (Fig. 7D), indicating that caspase activation had occurred. These results indicate that the release of cyt-c during simulated I/R is not inhibited by antiapoptotic proteins, and is not associated with Bax activation and translocation to the mitochondria.
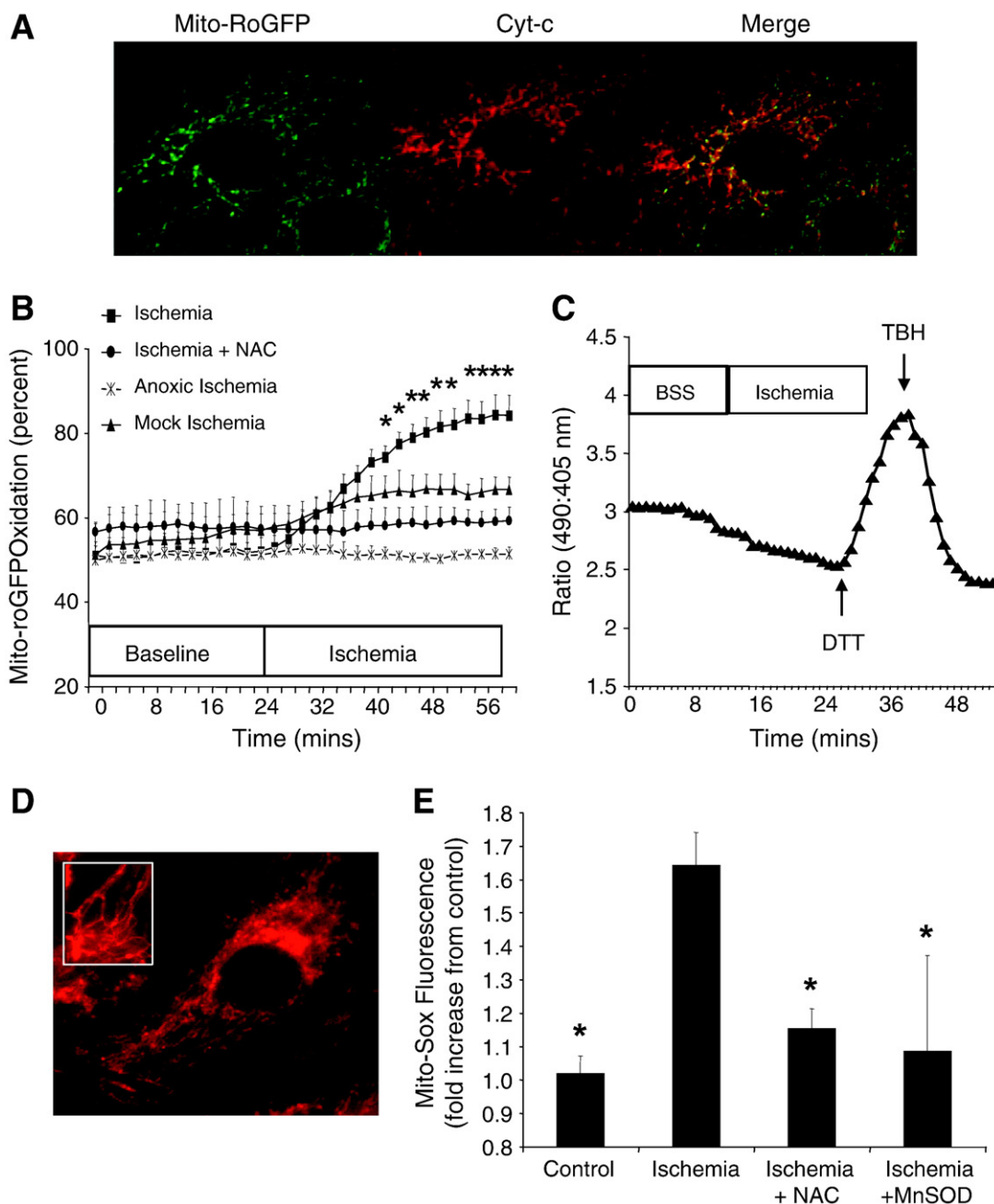
Fig. 3. Detection of oxidant stress in the mitochondrial matrix during simulated ischemia. (A) Isolated cardiomyocytes were transduced with an adenovirus to express mito-roGFP in the mitochondrial matrix. Cytochrome c immunostaining and merged images demonstrate the expected co-localization within the mitochondria. (B) Ratiometric measurements made using the mito-roGFP sensor showed a significant increase in the oxidation state within the mitochondrial matrix during simulated ischemia. This oxidation response was attenuated during "mock ischemia" (20% CO_2, 21% O_2, 59% N_2), anoxic ischemia (PO_2 <0.5 mm Hg), or ischemia in the presence of NAC (500 μmol/L) (mock ischemia, anoxic ischemia, ischemia + NAC, n = 3; Controls, n = 4; *p < .05). (C) Representative tracing of ratiometric measurements shows the dynamics of the mito-roGFP sensor in cardiomyocytes during simulated ischemia, followed by calibration of the sensor using dithiothreitol (DTT, 1 mmol/L) followed by t-butyl hydroperoxide (TBH 1 mmol/L). (D) Mito-Sox Red staining of the mitochondrial matrix in cultured cardiomyocytes. (E) Increases in Mito-Sox Red fluorescence relative to baseline values were evident within 20 min of simulated ischemia. No significant increase was detected in control cells maintained under baseline conditions for 20 min (Control) or in cells exposed to ischemia in the presence of NAC (Ischemia + NAC). Over-expression of Mn-SOD, induced using an adenoviral vector, significantly attenuated the rise in Mito-Sox fluorescence observed during simulated ischemia (Ischemia + NAC) (Control, n = 6; ischemia, n = 8; ischemia + NAC, n = 4; ischemia + Mn-SOD, n = 3; *p < .05).

3.8. Role of Bax/Bak and cytochrome c release in I/R-induced cell death

To further explore the requirement for apoptotic cell death in the setting of I/R, immortalized mouse embryonic fibroblast cells (MEFs) with genetic deletion of the proapoptotic proteins Bax and Bak (Bax$^{-/-}$/Bak$^{-/-}$ double knockout cells) were compared with immortalized MEFs from isogenic wild type animals. Wild type immortalized MEFs, immortalized Bax/Bak double knockout MEFs, and immortalized MEFs with stable over-expression of Bcl-X$_L$ were grown on glass cover slips

and subjected to serum withdrawal (48 h). Cell death in response to serum withdrawal was significantly attenuated in Bax/Bak-deficient cells, and by over-expression of Bcl-X$_L$ (Fig. 8A). In contrast, neither Bax/Bak deletion nor Bcl-X$_L$ over-expression conferred significant protection against simulated I/R.

As cytochrome c is released to the cytosol during simulated reperfusion (Fig. 6), we sought to clarify the significance of this event in I/R-induced cell death. Murine embryonic cells (ES cells) devoid of cytochrome c (cyt-c$^{-/-}$) were obtained from ATCC. Previous studies
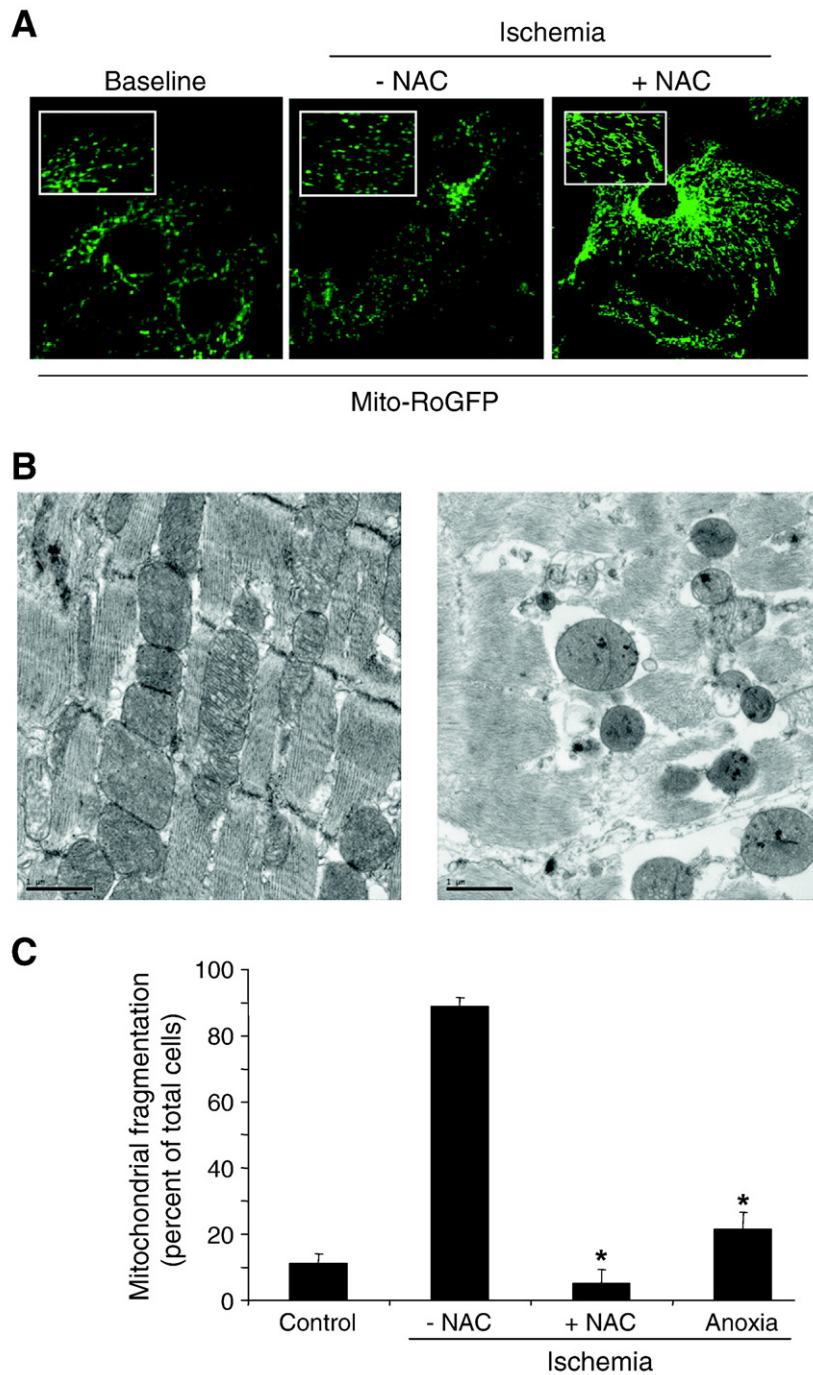
Fig. 4. Mitochondrial fragmentation in cardiomyocytes during simulated ischemia. (A) Cardiomyocytes were transduced with mito-roGFP and visualized under fluorescent microscopy (excitation: 484 nm, emission: 525 nm) to detect real time changes in mitochondrial morphology. Cells perfused under baseline conditions or subjected to simulated ischemia in the presence of N-acetylcysteine (NAC, 500 μmol/L) retained a normal reticular mitochondrial morphology. Cells exposed to ischemia in the absence of NAC demonstrated a significantly fragmented mitochondrial morphology. (B) Representative electron micrographs of mouse ventricular tissue from 400 μm slices maintained under baseline conditions (left) or after 30 min simulated ischemia without reperfusion (right). Bars are 1 μm in length. (C) Cardiomyocytes expressing mito-roGFP were subjected to baseline conditions or simulated ischemia with or without NAC or anoxia treatment. Images were analyzed by 2 independent observers in order to quantify the number of cells with fragmented mitochondria, as a percent of the total cells per high power field (50–70 cells from 3 distinct regions per cover slip). NAC and anoxic ischemia using Oxyrase both significantly reduced the percentage of cells with fragmented mitochondria. (n = 3 each; *p < .05 compared with Ischemia − NAC).

demonstrated that these cells are resistant to various apoptotic stimuli including UV irradiation, staurosporine, and serum withdrawal [31]. Cytochrome c-deficient ES cells and murine wild type (WT ES cells) were grown on cover slips and subjected to simulated I/R. However, deletion of cytochrome c did not confer protection against I/R (Fig. 8B). These findings indicate that, although apoptosis is activated during simulated I/R as evidenced by cytochrome c release

and DNA laddering, cell death does not require cytochrome c release in this model.

3.9. Temporal relationship between PI uptake and cytochrome c release

HeLa cells stably expressing a fusion protein consisting of cyt-c and GFP [18] were used to assess the temporal relationship between

cytochrome c release to the cytosol and propidium uptake, during simulated I/R. Co-localization studies using Mitotracker Red and GFP fluorescence demonstrated significant co-localization under confocal microscopy (Fig. 9A). During ischemia, there was minimal release of cyt-c and minimal uptake of PI in these cells (Fig. 9B). Within 1 h after reperfusion, significant PI uptake was observed, indicative of plasma membrane disruption. In contrast, a progressive increase in cyt-c release was observed during 3 h of reperfusion. These findings suggest that cyt-c release is not the primary mechanism driving cell death in this model.

4. Discussion

Oxidant stress and mitochondrial apoptosis have both been implicated in the cell death observed during I/R, but the relationship between these contributing factors is not clear. A substantial body of evidence indicates that ROS production occurs during I/R [46,52], and that chemical antioxidants confer protection against I/R-induced cell death [30]. However, apoptosis also has been reported to be activated by I/R, and to be important for mediating I/R-induced cell death [23,43]. It is also conceivable that oxidant stress during I/R mediates
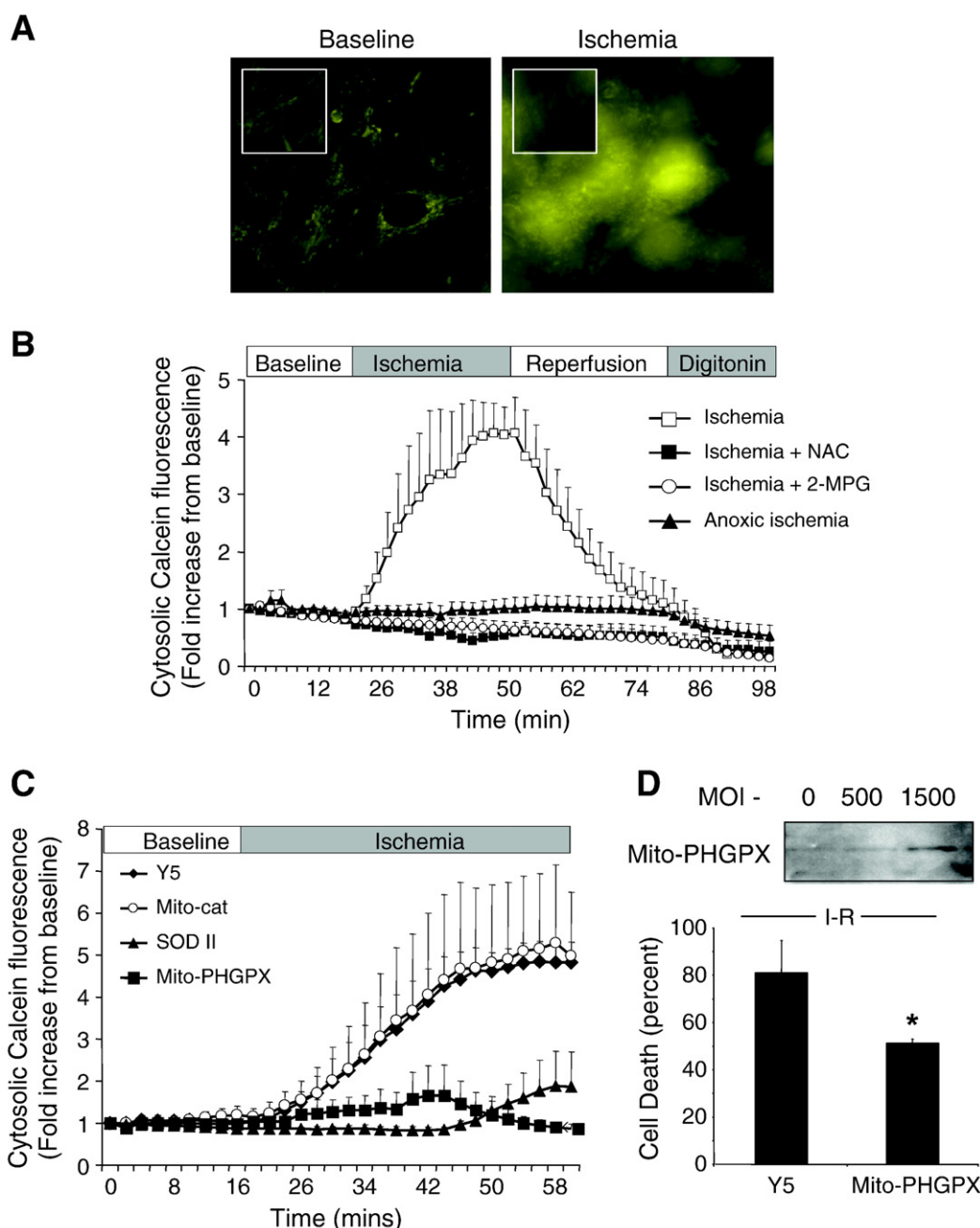

Fig. 5. Monitoring the opening of the mitochondrial permeability transition pore (mPTP) during simulated ischemia using mitochondrial calcein fluorescence. (A) Cardiomyocytes were loaded with calcein dye (1 mmol/L) in the presence of cobalt chloride (CoCl$_2$ 1 mmol/L) for 30 min and then studied in the flow-through chamber. During baseline perfusion a mitochondrial pattern of calcein fluorescence was observed. Within 16 min after beginning simulated ischemia, a rapid and profound release of calcein to the cytosol was observed. (B) A significant increase in calcein fluorescence intensity was detected during simulated ischemia. This increase was significantly attenuated by NAC (500 μmol/L) or 2-MPG (400 μmol/L) administration prior to and during ischemia. Anoxic ischemia using Oxyrase also prevented calcein release (2-MPG, n = 3; controls, NAC, anoxic ischemia, n = 4). (C) Adenoviral over-expression of mitochondrial phospholipid hydroperoxide glutathione peroxidase (mito-PHGPx, 1500 MOI) or manganese superoxide dismutase (Mn-SOD, 100 MOI) conferred significant protection against calcein escape from mitochondria during ischemia. Mitochondrial-targeted catalase (Mito-cat, 500 MOI) did not attenuate the rise in cytosolic calcein fluorescence (Controls, mito-PHGPx, n = 5; Mn-SOD, n = 6; Mito-cat, n = 4). (D) Over-expression of mito-PHGPx also conferred significant protection against cell death after 1 h of ischemia and 3 h of reperfusion, compared with cells transduced with Y5 empty virus (n = 5; *p<0.05).
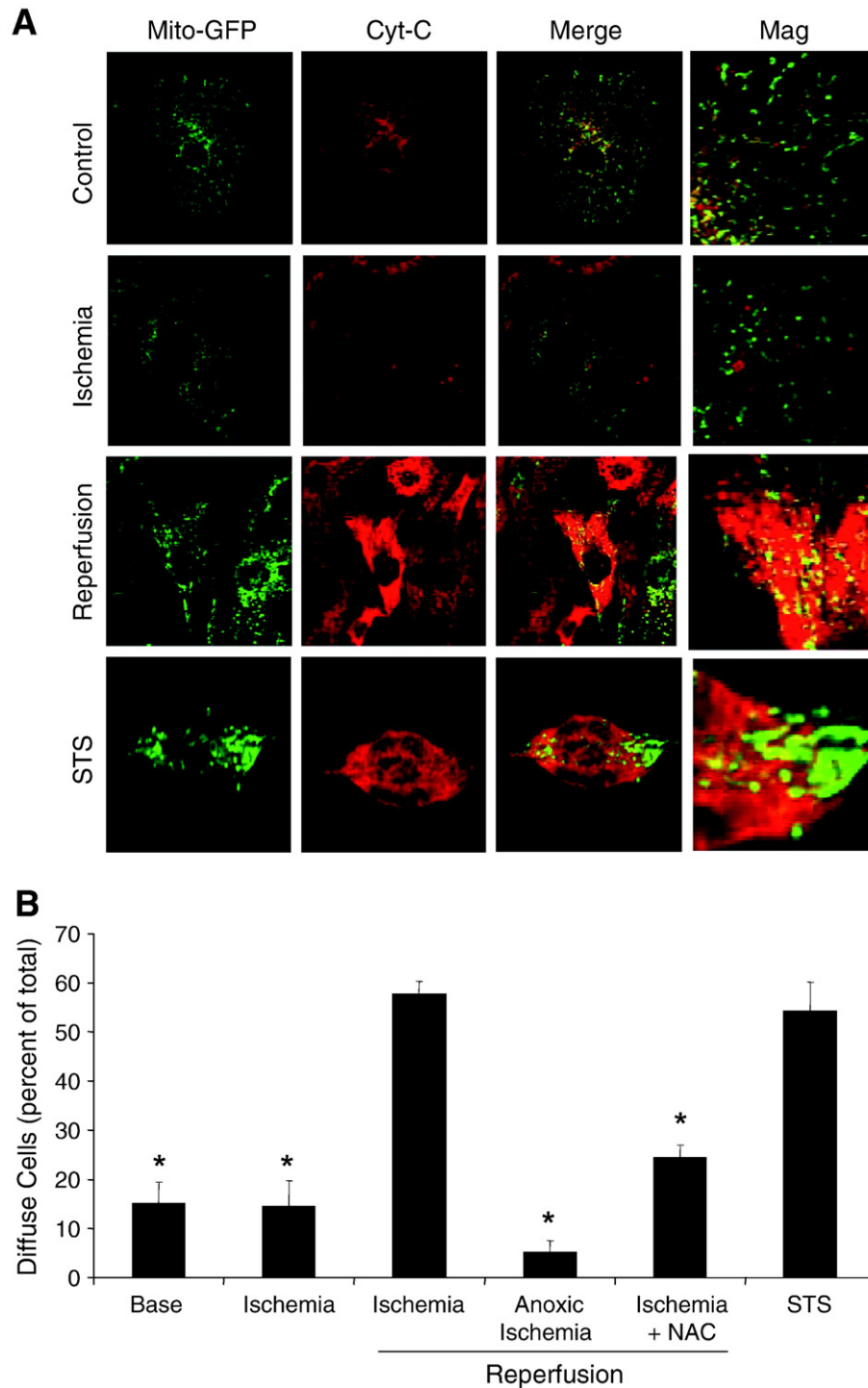
Fig. 6. Release of cytochrome c during simulated I/R. (A) Cardiomyocytes were transduced with mito-roGFP for 48 h to identify the mitochondria. Cells were fixed at baseline, after 45 min of ischemia, or after 2 h of reperfusion. Cells were then immunostained for cytochrome c to assess the degree of co-localization with the mito-roGFP sensor. Significant redistribution of cytochrome c to the cytosol was noted after 2 h of reperfusion, while only trace levels of redistribution were detected during ischemia (mag. 60×). (B) Cells showing redistribution of cytochrome c were enumerated and expressed as a percent of the total cell population. Staurosporine (STS) caused a significant increase in the percentage of cells with cytochrome c release. A similar increase was seen in cells subjected to simulated I/R. Cytochrome c release was attenuated in cells treated with NAC prior to and during ischemia, and in cells challenged with anoxic ischemia using Oxyrase. No significant difference at baseline or after (base, ischemia, ischemia/reperfusion, STS, $n = 4$; NAC, anoxic ischemia, $n = 3$; $*p < 0.05$).

cell death by activating a programmed cell death pathway [40]. We previously reported that oxidant stress generated during simulated ischemia is required for cell death during reperfusion [41]. The present study sought to examine how oxidant stress generated during ischemia contributes to cell death, and the relationship between ischemic oxidant stress and the activation of mitochondrial apoptosis.

4.1. Ischemia causes oxidant stress in the mitochondrial matrix during simulated I/R

We utilized mito-roGFP, a ratiometric redox sensor, to detect protein thiol oxidation during ischemia in the mitochondrial matrix. During simulated ischemia, oxidation of this sensor increased from

less than 50% to more than 90%, indicating that mitochondrial ROS production increases paradoxically during ischemia, before experiencing the ROS burst that occurs upon reperfusion. Ischemic oxidant production was abrogated by chemical antioxidants, and by scavenging all of the residual O_2 from the ischemia media to prevent ROS generation, indicating that ROS were involved. Mitochondrial ROS production was also detected using Mito-SOX, a superoxide-sensitive cationic dye that accumulates in mitochondria. Oxidation of Mito-SOX was attenuated by antioxidants and by Mn-SOD over-expression, indicating that superoxide is the initial oxidant generated in that compartment. A paradoxical increase in ROS production during ischemia was originally predicted by Misra and Fridovich, who postulated that the increase in reduction state of the mitochondrial electron carriers during hypoxia would promote ROS generation despite a decrease in the availability of O_2 levels [35]. We previously detected such oxidant stress in the cytosol of cardiomyocytes in our simulated I/R model [41]. The present finding extends that work by showing that oxidant stress increases in the matrix as well, suggesting that ischemic oxidants originate from the electron transport chain. It seems unlikely that each of the chemically diverse antioxidants was able to scavenge superoxide directly. More likely, some of these agents may have acted by protecting the redox status of proteins that were the targets of superoxide attack.

4.2. Ischemic oxidant stress is critical for cell death in simulated I/R

ROS production during ischemia is required for cell death during reperfusion. This conclusion is based on the observation that chemical antioxidants given during the ischemia were protective, as was anoxic ischemia achieved by O_2 scavenging. These findings are consistent with our previous work [41].

Ischemic oxidant stress causes a change in mitochondrial morphology, from a reticular to a punctate or fragmented appearance. In association with this morphological change, mitochondria release calcein from the matrix to the cytosol, a response that signals the formation of a large conductance pore in the mitochondrial membranes. The most likely source of this leak is the mPTP, which has previously been implicated in the depolarization of mitochondria during reperfusion after ischemia [10,13]. Antioxidants and anoxic ischemia prevented the calcein leak, as did over-expression of mito-PHGPx. This protein is targeted to the mitochondrial membranes and is responsible for scavenging lipid hydroperoxides [17]. Over-expression
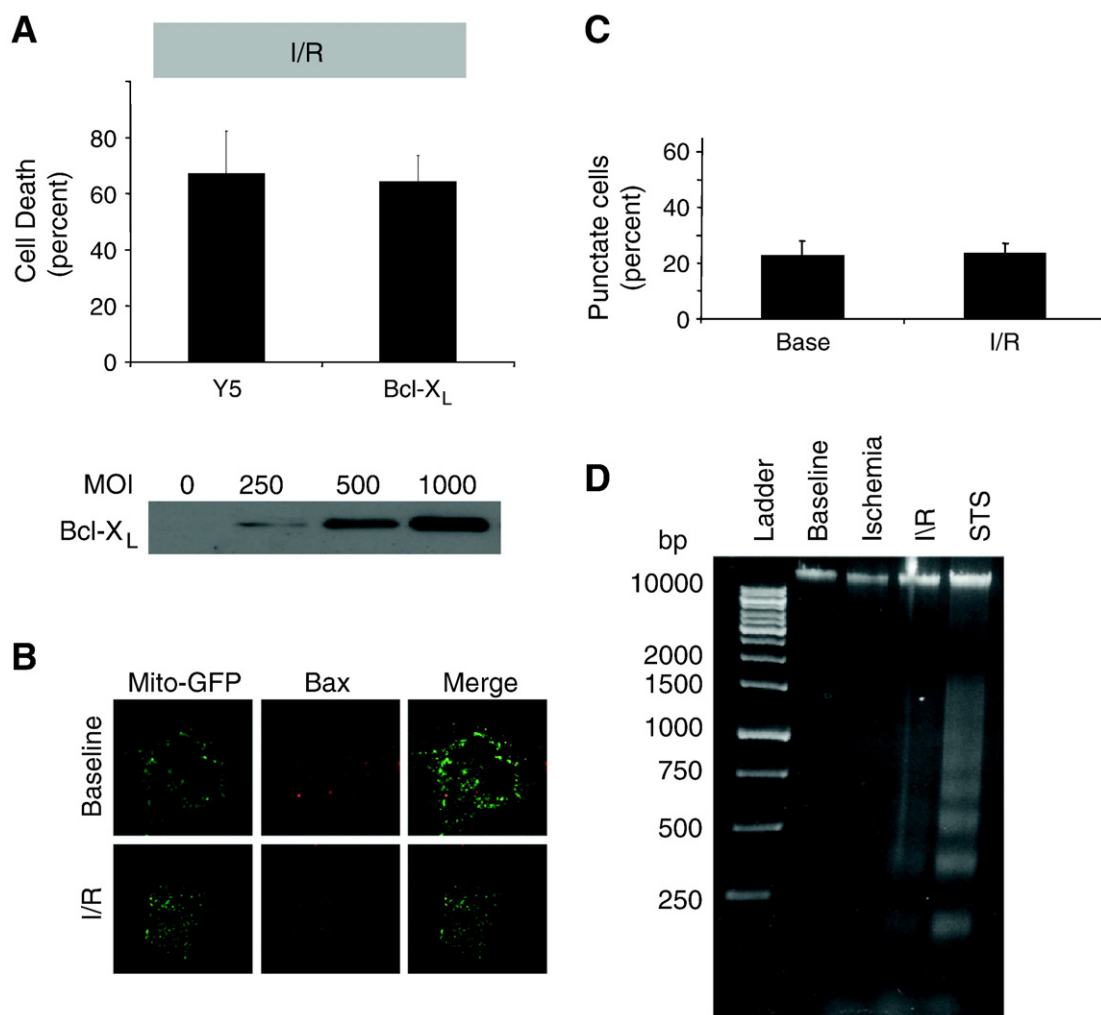

Fig. 7. Mitochondrial apoptosis in staurosporine-treated and I/R-challenged cardiomyocytes. (A) Bcl-X_L over-expression with an adenovirus (1000 MOI) for 36 h in cardiomyocytes was not protective against simulated I/R compared with cells transduced with empty virus (Y5, 1000 MOI) (n = 4). (B) Cardiomyocytes transduced with an adenovirus to express mito-roGFP for 48 h, in order to identify mitochondria. Cells were then subjected to simulated I/R and immunostained for Bax, in order to assess oligomerization as evidenced by co-localization with the mitochondrial fluorescence, by confocal microscopy. I/R did not induce significant Bax activation, as seen by the lack of co-localization in this representative image (mag. 60×). (C) No significant increase in the number of cells exhibiting a mitochondrial localization of Bax after simulated I/R was detected, indicating that I/R was not a significant stimulus for the activation of mitochondrial apoptosis (n = 4). (D) Representative gel showing the presence of DNA fragmentation after treatment with I/R (lanes: 1—Ladder, 2—Baseline, 3—Ischemia, 4—Ischemia + Reperfusion (I/R), 5—Staurosporine (STS)).
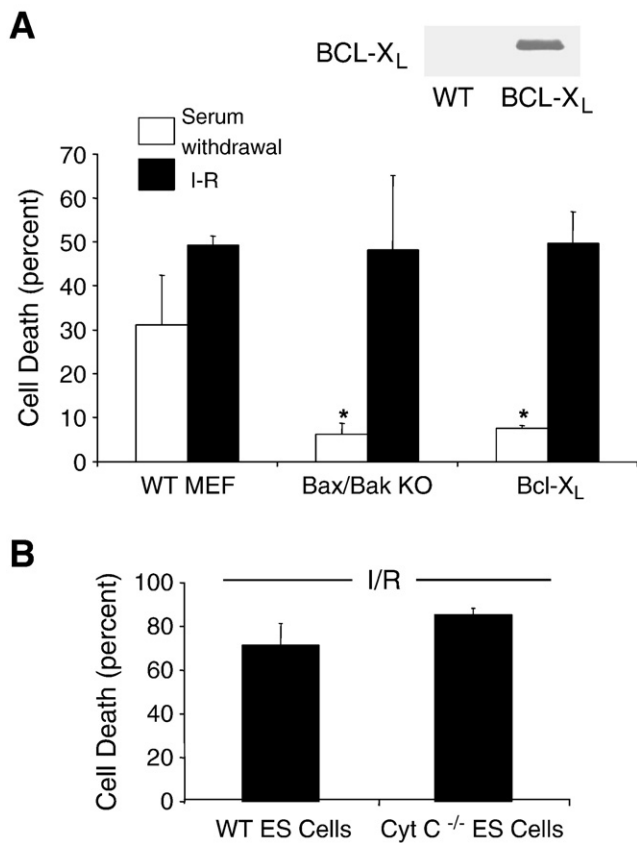
A

B

Fig. 8. Cell death in wild-type MEFs, Bax/Bak double knockout (KO) MEFs, Bcl-X$_L$ over-expressing MEFs, wild-type ES cells and cytochrome c deficient ES cells. (A) Bcl-X$_L$ over-expression and genetic deletion of Bax/Bak conferred protection against growth factor withdrawal for 72 h as assessed by LDH cytotoxicity assays. However, in cells subjected to 30 min of simulated ischemia followed by 3 h reperfusion there was no significant difference in cell death between groups as determined by PI uptake (n = 5; *p<.05 compared with controls). (B) Murine embryonic stem (ES) cells lacking cytochrome c (cyt-$c^{-/-}$) were not significantly protected against 60 min of simulated ischemia followed by 3 h of reperfusion compared with wild-type ES cells as shown by analysis of PI uptake (n = 4).

of mito-PHGPx conferred protection against I/R-induced cell death, and it abrogated the ischemia-induced calcein leakage. These results suggest that mitochondrial oxidant stress is responsible for triggering the mPTP opening, which is responsible for the mitochondrial depolarization and cell death. A previous report also noted that mito-PHGPx over-expression was protective in I/R [24].

Protection against calcein leak and cell death was also conferred by Mn-SOD, which suggests that superoxide production in the mitochondrial matrix is critical for regulating cell death in this model. This finding is consistent with studies showing that protection from hepatic and cardiac I/R injury *in vivo* is conferred by over-expression of Mn-SOD in mice [8]. Loss of this critical antioxidant in mice causes a severe dilated cardiomyopathy and neonatal lethality [28,32], underscoring the potential toxicity of intra-mitochondrial superoxide. Recently, Ago et al. reported that NOX4 may be expressed in mitochondria, and that over-expression of NOX4 can lead to cell death in aging and hypertrophic hearts [1]. While the electron transport chain has been regarded as the apparent source of superoxide in ischemia, those findings raise the question of whether NOX4 contributes importantly to the generation of superoxide during ischemia. In either case, superoxide appears to be critical for the cell damage, as catalase expression in either the cytosol or in the mitochondrial matrix failed to provide protection, which indicates that H_2O_2 is a less important factor in this model. Although there is little doubt that excessive H_2O_2 can potentially injure a cell, our findings do suggest that superoxide in the mitochondrial matrix may

act on different targets than does H_2O_2, with important consequences for the rapid initiation of cell death pathways. Over-expression of Cu, Zn-SOD provided no significant protection in this model. Hydrogen peroxide generated in one intracellular compartment can diffuse through membranes to access other compartments. In contrast, superoxide generated in I/R cannot easily cross membranes, which explains why Mn-SOD was protective in the I/R model whereas Cu,Zn-SOD was not.

Previous studies have suggested that mPTP opening only occurs after reperfusion in intact heart, as the low pH during the ischemic insult inhibits pore activation [11,21,22]. Although our cellular model of simulated ischemia includes hypercarbic acidosis, this did not prevent activation of the pore during the ischemia. Despite this difference, important similarities are seen between this model and the intact heart, including the ability to undergo preconditioning protection [45], involvement of ROS in the cell death [30], and the development of cell death after reperfusion [47]. Further studies are needed to determine whether more severe acidosis would delay the onset of mPTP opening until after the start of reperfusion.

4.3. Relationship between mPTP activation and cytochrome c release

Previous studies of I/R have shown that mPTP opening at reperfusion is associated with cytochrome c release to the cytosol [11,12]. Our findings confirm that negligible cyt-c release occurs before reperfusion, yet our results consistently show that calcein release occurs in parallel with mitochondrial depolarization during ischemia, in an ROS-dependent manner. One possibility is that mPTP activation during ischemia represents oscillation or flickering of the pore, whereas irreversible opening occurs at reperfusion. Our findings are consistent with the mPTP oscillations reported by Di Lisa and colleagues, who concluded that cell death was not triggered by these short mPTP openings [39]. The only difference between their findings and ours is that we observed release of both calcein and TMRE during this oscillation, whereas they observed release of calcein but not TMRM. In either case, our findings suggest that the ROS-mediated oscillation of the mPTP during ischemia is an important determinant of later reperfusion-induced cell death. We agree with their conclusion that mPTP oscillation by itself is not lethal for the cell, based on our observation that cyt-c release and cell death do not increase significantly until reperfusion, even when the ischemic insult is extensively prolonged.

4.4. Role of mitochondrial apoptosis in cell death following I/R

Simulated I/R caused a redistribution of cyt-c from the intermembrane space to the cytosol. This, plus the associated DNA laddering, suggests that caspases were activated by that event. However, over-expression of Bcl-X$_L$ in cardiomyocytes and MEFs failed to induce protection, and there was no evidence of Bax oligomerization in cardiomyocytes during I/R. Furthermore, MEFs derived from Bax/Bak double knockout embryos were not protected against I/R, but did show protection against serum withdrawal, a potent stimulus for activating mitochondrial apoptosis. These findings indicate that activation of BH-domain proteins is not required for I/R-induced cell death.

4.5. Activation of redundant death pathways mediated by mPTP oscillation

Although cytochrome c release occurs in response to I/R, our data suggest that this is a marker rather than a mediator of cell death. First, genetic depletion of cytochrome c was not protective against cell death in this model. Second, when comparing the kinetics of PI uptake and the release of cyt-c, it is apparent that PI uptake occurs within the first hour after reperfusion whereas cytochrome c release develops
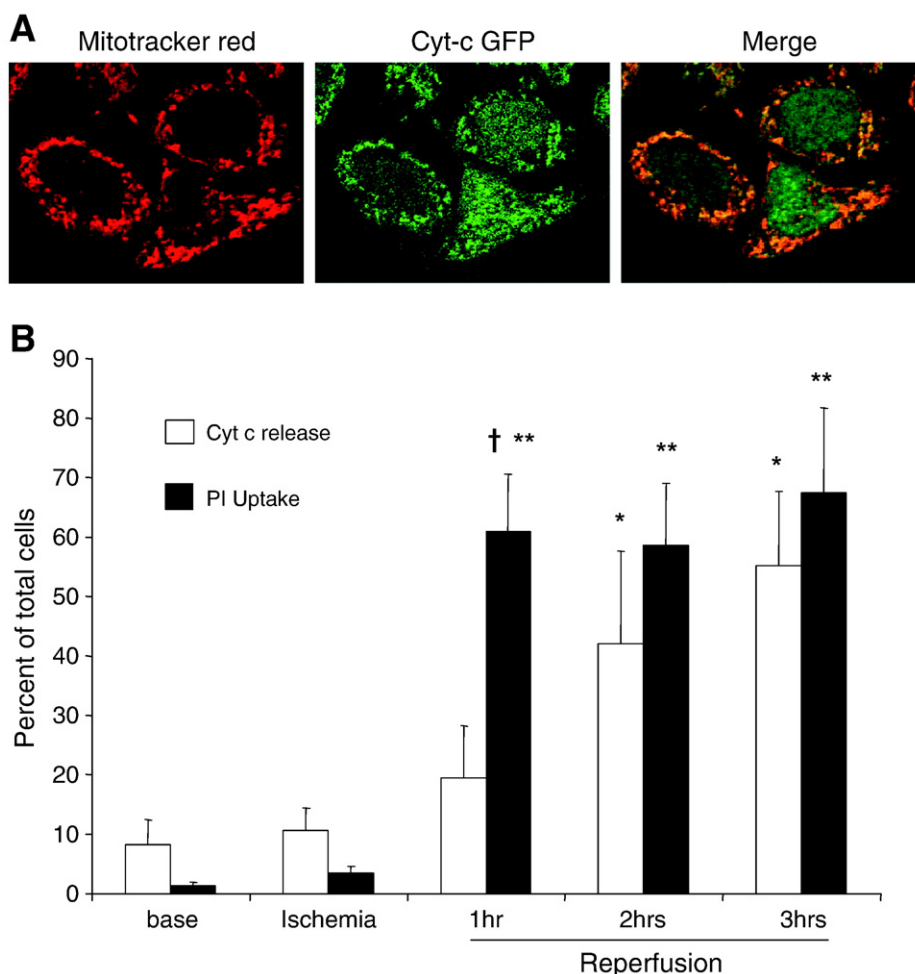
Fig. 9. Cytochrome c release and PI uptake during simulated I/R in HeLa cells with stable expression of cytochrome c-GFP. (A) Cytochrome c-GFP-expressing HeLa cells were loaded with Mitotracker Red (100 nM) for 30 min to label mitochondria. Merged confocal image shows co-localization of the cytochrome c-GFP fluorescence and Mitotracker Red (mag. 60×). (B) HeLa cells challenged with simulated I/R. Live cell images were obtained during baseline, ischemia, and during reperfusion. Rapid and extensive dissipation of punctate cyt-c-GFP fluorescence was observed within 2 h of reperfusion. In separate experiments, propidium iodide was used to detect the extent of cell death in the HeLa cells during simulated I/R. Significantly greater cell death within 1 h of reperfusion was observed using PI uptake compared with cellular cytochrome c redistribution. The percentage of cells demonstrating cytochrome c release approximated the percentage of cells exhibiting PI uptake by 2 h of reperfusion. (Cyt-c redistribution and PI uptake, n = 4; *p < 0.05 compared to cyt-c release at baseline; **p < 0.05 compared to PI uptake at baseline; †p < 0.05, PI uptake compared to percent of cells with diffuse cyt-c staining).

later. This suggests that cytochrome c release is not required for the loss in plasma membrane integrity. Rather, events early in reperfusion, most likely the ROS burst, appear to be responsible for triggering both the loss of plasma membrane integrity and the later release of cytochrome c. Once the plasma membrane integrity is lost, a cell becomes committed to death regardless of whether or not cytochrome c is later released. Our data suggest that low levels of oxidant stress during ischemia trigger oscillation of the mPTP, which causes cell death during reperfusion, possibly by initiating a lethal ROS burst. Although the specific mechanism is not fully understood, we speculate that mPTP activation during ischemia may prime the cell for undergoing a burst in oxidant stress at reperfusion by releasing NAD(P)H from the matrix to the cytosol. Di Lisa and colleagues demonstrated the feasibility of such a process by showing that mitochondrial NAD$^+$ release to the cytosol occurs in response to mPTP activation [13]. A burst of ROS generation at reperfusion, resulting from the oxidation of mitochondria-derived NADH in the cytosol, could then trigger plasma membrane disruption and also cause irreversible mPTP opening and cytochrome c release. In that respect, oxidant stress during ischemia would represent a causative factor in the lethal ROS burst during reperfusion, which would undermine cell survival by activating multiple redundant death pathways. A fuller test of this hypothesis is warranted.

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