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Original Article

Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome *c* release and apoptosis induced by heart ischemia

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Abstract

Ischemia/reperfusion of heart causes contractile dysfunction, necrosis and/or apoptosis and is a major cause of human death, but the molecular mechanisms are unclear. We show that ischemia alone (without reperfusion) is sufficient to induce apoptosis and mitochondrial dysfunction, and we have investigated the mechanism responsible; 30 and 60 min stop-flow ischemia in Langendorff-perfused rat hearts induced progressive (a) release of cytochrome *c* from mitochondria to cytosol, (b) inhibition of the mitochondrial respiratory functions, (c) activation of caspase-3-like protease activity and (d) DNA strand breaks (however, only 2% of myocyte nuclei were TUNEL positive at 60 min). Fifteen minutes pre-perfusion of hearts with cyclosporin A, an inhibitor of mitochondrial-permeability transition (MPT), largely prevented all these ischemic changes. Pre-perfusion of hearts with FK506, an inhibitor of calcineurin, caused no protection. Pre-perfusion with DEVD-CHO, an inhibitor of caspase-3-like proteases, completely prevented ischemia-induced DNA strand breaks, but only partially blocked cytochrome *c* release and mitochondrial respiratory inhibition. Reperfusion of hearts after 30 min ischemia further stimulated caspase activity and nuclear apoptosis. We conclude that ischemia-induced MPT causes release of cytochrome *c*, which then activates the caspases that execute apoptosis and feedback to cause further cytochrome *c* release. The MPT-induced cytochrome *c* release is also largely responsible for the ischemic respiratory inhibition, which might contribute to contractile dysfunction or necrosis at reperfusion.

Keywords: Ischemia; Apoptosis; Mitochondrial-permeability transition; Cytochrome *c*; Respiration; Caspases

1. Introduction

Recent evidence indicates that ischemia/reperfusion can induce apoptosis in heart myocytes both in animals and humans [1–4], as indicated by DNA laddering, DNA fragmentation and/or caspase activation. However, the mechanism by which apoptosis is induced by ischemia and/or reperfusion is still unclear. Apoptosis is executed by a family of cysteine proteases-caspases, which can be activated by two main pathways (see Ref. [5] for review). The first pathway is initiated by binding of extracellular ligands (Fas ligand, TNF-α) to their cell-surface receptors leading to caspase-8 activation. The second is mediated by mitochondria, which release apoptotogenic proteins, mainly cytochrome *c*. Cytochrome *c* once released into the cytosol binds to a cytosolic protein Apaf-1, and this complex in the presence of dATP or ATP facilitates activation of caspase-9, which in turn activates downstream caspase-3. While these pathways have been defined in cultured cells undergoing apoptosis induced by various stimuli, little is known as to which caspase-activation pathway is triggered in the ischemic/reperfused heart. We and others have shown that ischemia/reperfusion of heart induces both cytochrome *c* release and caspase activation [2,6,7], but it is unclear whether cytochrome *c* release causes caspase activation or caspase activation causes cytochrome *c* release.

At least two ways of inducing cytochrome *c* release from mitochondria have been suggested by translocation from cytosol to mitochondria of BH3-domain-containing pro-apoptotic proteins (such as Bid, Bax or Bad) [8–10], or by mitochondrial-permeability transition (MPT) [11,12]. MPT can be triggered in response to stress signals, such as high

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Ca^{2+} or reactive oxygen species [13–16], conditions that are likely during ischemia/reperfusion. It is thought that MPT results from opening of a pore (the MPT pore) in mitochondrial membranes, which is formed by several proteins (including, probably, the voltage-dependent anion channel, ATP/ADP-translocator, cyclophilin D and creatine kinase) [13]. Upon MPT pore opening, the mitochondrial membrane potential collapses and proteins from the intermembrane space (including cytochrome *c*) can be released [11,17]. There is evidence that the MPT pore may open during reperfusion [15,18,19]; however, it is unclear whether this may occur during ischemia itself. In addition to apoptosis, heart ischemia/reperfusion induces myocyte necrosis and contractile dysfunction after reperfusion. The mechanisms by which necrosis is induced are also unclear, but have been suggested to include mitochondrial respiratory inhibition, cellular ATP depletion, Ca^{2+} influx, ROS production, MPT and hypercontracture [20,21]. Irreversible respiratory inhibition has partly been attributed to cytochrome *c* release [22], and may contribute to necrosis and contractile dysfunction after reperfusion.

In the research reported here, we show that ischemia alone (without reperfusion) can induce cytochrome *c* release, mitochondrial dysfunction, caspase activation and nuclear apoptosis, and that all these ischemic events can be prevented by blocking the MPT pore.

2. Methods

Hearts from male Wistar rats were used in the experiments. Hearts were perfused using a Langendorff-perfusion system with Krebs–Henseleit solution (11 mM glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.7 mM $MgSO_4$ and 0.7 mM Na pyruvate; pH 7.2 at 37 °C), with or without 0.1 μM cyclosporin A (Sigma), 25 μM FK506 (Calbiochem) or 1 μM DEVD-CHO (cell permeable caspase-3 inhibitor with N-terminal sequence, which facilitates the peptide's cell permeability; Biomol). After 15 min of perfusion, stop-flow ischemia was induced for 30 or 60 min.

The apoptotic cells were identified by dUTP nick end labelling (TUNEL) using CardioTACS (R&D Systems) apoptosis detection kit according to the manufacturer's protocol. Heart ventricular tissues were fixed in 3.7% formaldehyde for 24 h, embedded in paraffin and sliced. TUNEL-positive cells were counted using a light microscope. Cardiomyocytes were identified by typical morphological features and striations, clearly seen at 1000× magnification; 8–20 slices were analyzed from each heart ventricle, each slice contained about 450 myocytes.

Mitochondria were isolated by standard procedures as described previously [22], except that isolation buffer contained 0.1 μM cyclosporin A and 2 mM EGTA. Mitochondrial respiration was measured with a Clarke-type oxygen electrode in 1 ml of incubation medium-containing 110 mM KCl, 10 mM Tris–HCl, 5 mM KH_2PO_4, 50 mM creatine, 1 mM $MgCl_2$, and either 1 mM pyruvate + 1 mM malate or 10 mM succinate (+1 μM rotenone); pH 7.2. Mitochondrial state 3 respiration rate was obtained by adding 1 mM ATP, which was converted to ADP by creatine kinase (4 IU/ml). In some experiments, mitochondrial respiration was measured in the presence of 30 μM exogenous cytochrome *c*. Membrane potential was measured using a tetraphenylphosphonium-selective electrode, as described previously [22]. Kinetics of the respiratory chain was determined as the dependence of the mitochondrial respiration rate on the membrane potential, when the latter was titrated with carboxyatractyloside (0.4–8 nmol/mg mitochondrial protein).

Skinned cardiac fibers were prepared as described in Ref. [23]. Briefly, bundles of the heart muscle fibers (0.2–0.3 mM in diameter) were prepared and transferred to buffer A, containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 7.1 mM $MgCl_2$, 50 mM 2-[*N*-Morpholino]ethanesulfonic acid (MES), 5 mM ATP, 15 mM phosphocreatine, 2.6 mM CaK_2EGTA and K_2EGTA (free Ca^{2+} concentration 0.1 μM); pH 7.0, supplemented with 50 mg/ml saponin (from *Gypsophila*; Sigma) and 900 U/ml collagenase (from *Clostridium hystolyticum*, type IV, Sigma) and incubated for 30 min. Then, the bundles were washed for 10 min in buffer B, containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM $MgCl_2$, 100 mM MES, 3 mM CaK_2EGTA and 7.1 mM K_2EGTA; pH 7.1. Oxygen uptake rates of skinned fibers were measured in buffer B supplemented with 2 mg/ml bovine serum albumin (Fraction V, Sigma) and 1 mM ADP. To measure levels of cytochromes *c* and *a* mitochondria were solubilized with 1% Triton X-100. Sodium hydrosulfite-reduced minus hydrogen-peroxide-oxidized absorption difference spectra were recorded with a Hitachi-557 spectrophotometer, and content of mitochondrial cytochromes was calculated as described in [24]. Cytochrome *c* and cytochrome oxidase (COX) in cytosolic fractions of the hearts were identified by western blotting. Samples of cytosolic fractions were separated by 15% SDS-PAGE and blotted onto nitrocellulose membrane. Proteins were identified using primary monoclonal antibodies to denaturated cytochrome *c* (PharMingen) or to COX subunit IV (Molecular Probes) and secondary antibodies conjugated with horseradish peroxidase (Jackson ImMunoResearch Laboratories) and detected using ECL western blotting detection reagents (Amersham Pharmacia Biotech). Activity of caspases was measured in cytosolic fractions isolated from heart homogenates in sucrose-based medium (250 mM sucrose, 5 mM HEPES, 2 mM EGTA, 1 mg/ml bovine serum albumin) by differential centrifugation: 5 min × 750 *g*, 10 min × 6800 *g*, and 60 min × 27200 *g* to obtain cytosolic fraction; 1 mg/ml of total cytosolic protein was incubated for 1 h in buffer-containing 10% sucrose, 50 mM HEPES, 1 mM $MgCl_2$, 1 mM ATP (pH 7.4, 37 °C) and 0.1 mM z-DEVD-*p*-nitroanilide, a caspase-3 substrate. The hydrolysis of caspase substrate was followed spectrophotometrically at 405 nm and was calibrated with *p*-nitroanilide. DEVD-cleaving activity was completely suppressed by 20 μM DEVD-CHO, an

inhibitor of caspase-3. Data are expressed as mean ± S.E. of 3–7 separate experiments. Statistical comparison between experimental groups was performed using Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Cyclosporin A prevents loss of cytochrome c *from mitochondria during myocardial ischemia*

We have previously shown that ischemia causes the release of mitochondrial cytochrome *c* in isolated rat heart [7,22]. To test whether this cytochrome *c* release is due to MPT, we pre-perfused hearts with a MPT inhibitor cyclosporin A (0.1 μM), then induced ischemia and measured the cytochrome *c* content of subsequently isolated mitochondria. Fig. 1 shows the effects of ischemia and cyclosporin A on mitochondrial content of cytochromes *c* and *a*. After 30 and 60 min ischemia, the mitochondrial level of cytochrome *c* decreased by about 30% and 50%, respectively, compared to control level. However, in hearts pre-perfused with cyclosporin A, there was no significant decrease in cytochrome *c* after ischemia. Cyclosporin A is an inhibitor of MPT, but it also can inhibit calcineurin. To test whether calcineurin might be involved in ischemia-induced loss of cytochrome *c* from mitochondria, hearts were pre-perfused with FK506, an inhibitor of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, which does not affect MPT. In contrast to cyclosporin A, FK506 had no effect on the decrease of mitochondrial cytochrome *c* during ischemia (Fig. 1A). Ischemia (and both inhibitors) had no effect on the mitochondrial content of cytochrome *a*, an integral component of the inner membrane (Fig. 1B). We also measured cytochrome *c* levels in cytosols using western blots. As can be seen in Fig. 1C, 30 min ischemia led to a substantial increase in cytochrome *c* in cytosols, and this was prevented when hearts were loaded with cyclosporin A prior to induction of ischemia. Cytochrome *c* oxidase subunit IV (COX), a marker for intactness of the mitochondrial inner membrane, was undetectable in all samples using the same amount of total protein as for detection of cytochrome *c* (data not shown) and was visible only when total protein was increased ~8 times (Fig. 1C). COX level was unchanged in cytosols from control, ischemic or ischemic + cyclosporin A hearts (Fig. 1C), indicating that there was no gross mitochondrial damage during ischemia or differential damage during the isolation procedure. The possibility of permeability transition occurring during the mitochondrial isolation procedure itself, rather than the previous ischemic period, was greatly reduced by including EGTA and cyclosporin A in the cold isolation medium [19]. Together, these results indicate that during ischemia, there is a specific, MPT-related release of cytochrome *c* from mitochondria into cytosol.

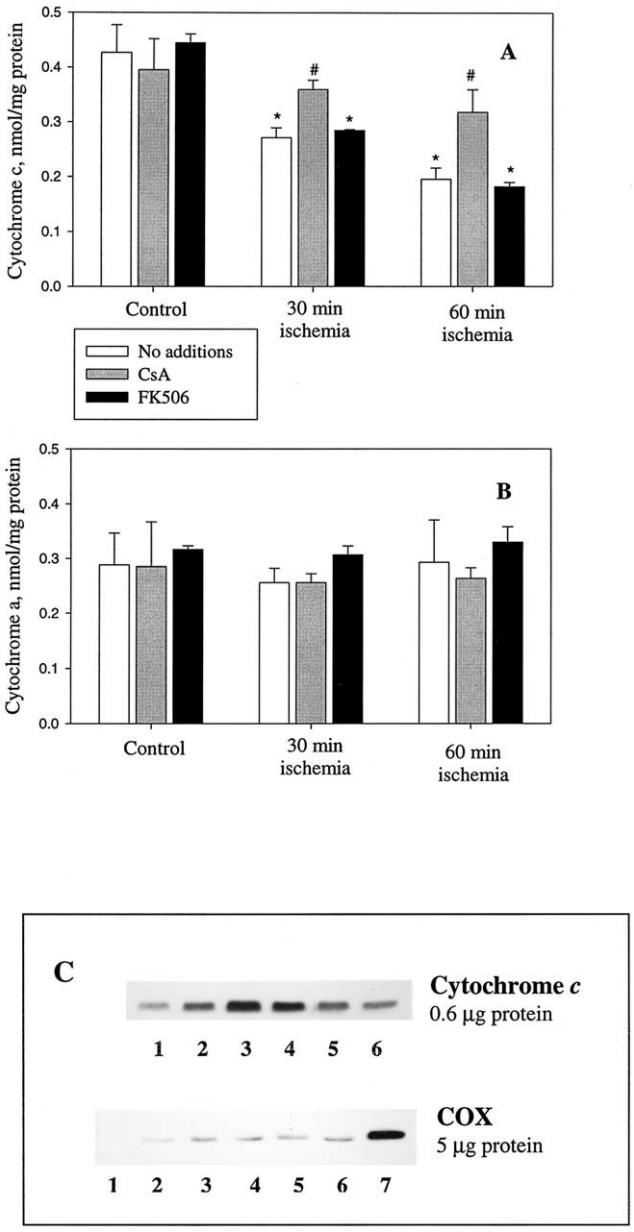

Fig. 1. Cyclosporin A protects heart mitochondria from loss of cytochrome *c* during ischemia. (A) Mitochondrial content of cytochrome *c*. (B) Mitochondrial content of cytochrome *a*. Hearts were pre-perfused for 15 min with 0.1 μM cyclosporin A, 25 μM FK506 or just with buffer (control) prior to induction of ischemia. Content of cytochromes *c* and *a* was measured spectrophotometrically in isolated mitochondria. (C) Western blots of cytochrome *c* and COX in cytosolic extracts from control and ischemic hearts. Lanes 1, 2—samples from control hearts; lanes 3, 4—samples from 30 min ischemic hearts; lanes 5, 6—samples from 30 min ischemic hearts treated with cyclosporin A. Mitochondrial protein of 5 μg was loaded in lane 7 for the COX assay to demonstrate that COX antibodies bind to mitochondrial COX effectively. Total protein of 0.6 μg was loaded in each lane for imMunoblotting of cytochrome *c* and 5 μg for COX. Two of six representative blots for each condition are shown. *—statistically significant effect of ischemia ($P < 0.01$), if compared to control; #—statistically significant effect of cyclosporin A ($P < 0.05$), if compared to treatment without cyclosporin in the same group (30 or 60 min ischemia).

3.2. Cyclosporin A reduces ischemia-induced mitochondrial dysfunction

Further experiments were designed to test whether MPT might be the cause of mitochondrial dysfunction induced by ischemia. The kinetics of the respiratory chain were measured in isolated heart mitochondria as the dependence of the mitochondrial respiratory rate on membrane potential, when this was titrated with an inhibitor of the ATP/ADP-translocator, carboxyatractyloside. The resulting curves (e.g. control in Fig. 2A) show the dependence of mitochondrial respiratory chain activity on membrane potential over the physiological range, and are independent of the mitochondrial ATP synthesis or proton leak activities [25]. Thus, changes in these kinetics indicate whether the activity of the respiratory chain is affected by the factors under investigation [25]. Fig. 2 shows that 30 and 60 min of ischemia caused a gradual but dramatic decrease in the activity of the respiratory chain, when succinate was used as substrate. This can be seen from the shift to the left (to lower respiratory rates) of the curve of the dependence of respiratory rate on membrane potential. Respiratory chain activity drops to about 50% of control after 30 min ischemia and to about 25% of control after 60 min ischemia, measured at the same membrane potential. However, in hearts pre-loaded with cyclosporin A, there was no significant decrease of respiratory chain activity after 30 min ischemia, and the decrease was much lower after 60 min ischemia, when compared to hearts not treated with cyclosporin A. Cyclosporin A had no effect on the kinetics of the respiratory chain when given to control (non-ischemic) hearts (Fig. 2A).

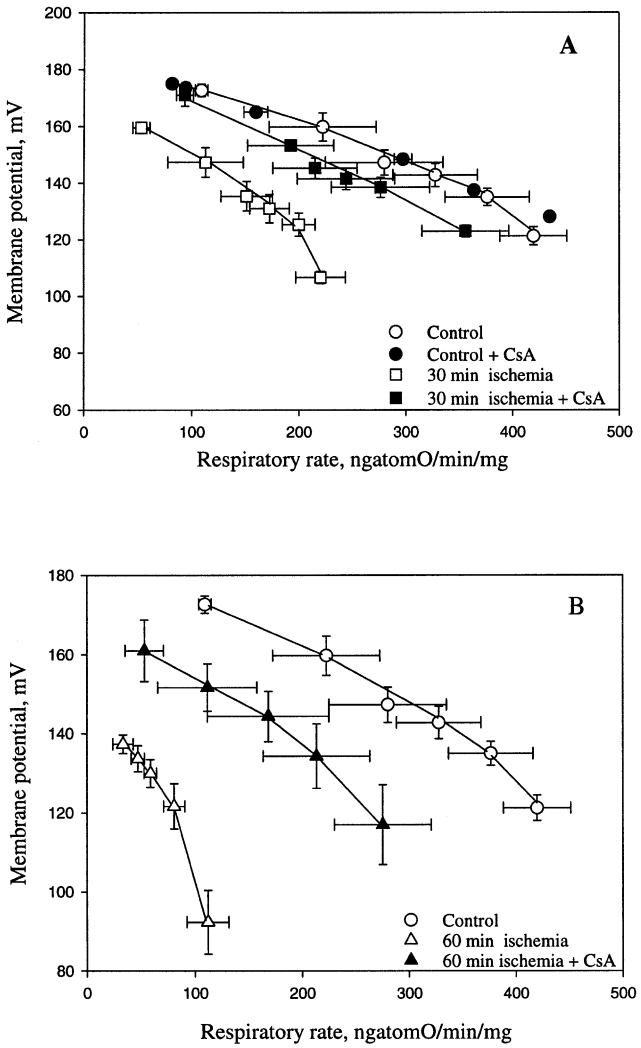

Fig. 2. Effect of cyclosporin A on the kinetics of the respiratory chain in mitochondria isolated from control and ischemic hearts. Pre-treatment of hearts with 0.1 μmol/l cyclosporin A reduces inhibition of the respiratory chain after (A) 30 min ischemia and (B) 60 min ischemia. Isolated mitochondria were placed in a vessel in which oxygen consumption and membrane potential were measured simultaneously; 10 mM succinate was used as substrate in the presence of 1 μM rotenone. Mitochondrial respiration rate and membrane potential were titrated with carboxyatractyloside (0.4–8 nmol/mg mitochondrial protein).

We also tested whether re-addition of cytochrome *c* to ischemia-damaged mitochondria would be sufficient to restore state 3 respiration rate. Exogenous cytochrome *c* stimulates respiratory rate, if there is mitochondrial outer membrane damage and a deficiency of endogenous cytochrome *c*. In these experiments, NADH-dependent respiratory substrates—pyruvate + malate—were used (thus, involving the Krebs cycle and complex I of the respiratory chain). As can be seen from Fig. 3A, 30–60 min ischemia caused a progressive decrease of the state 3 respiratory rate with pyruvate, which was largely prevented in the presence of cyclosporin A, but not FK506 (Fig. 3B). Addition of cytochrome *c* to 30 min ischemia-damaged mitochondria restored the respiratory rate close to control levels. After 60 min ischemia, addition of cytochrome *c* stimulated respiration by about 250% (Fig. 3C), but was not able to restore it to the control rate (Fig. 3B), suggesting that prolonged ischemia causes more severe damage to oxidative phosphorylation that is not only due to loss of cytochrome *c*. However, in mitochondria from 60 min ischemic hearts treated with cyclosporin A (in contrast to FK506 treatment), there was no significant ischemia-induced inhibition of respiration rate in the presence of cytochrome *c* (Fig. 3B). Exogenous cytochrome *c* increased the respiration of control mitochondria to a lesser extent, and this stimulation was similar in the hearts treated with cyclosporin A or FK506.

The procedure used to isolate mitochondria may affect the integrity of their membranes [26]. Therefore, we tested the effects of ischemia on the respiration and cytochrome *c* content of subsequently isolated skinned cardiac fibers, which are considered to contain morphologically and functionally intact mitochondria [27]. As can be seen from Fig. 4, there was a gradual decrease in state 3 respiration rate of fibers isolated from 30 and 60 min ischemic heart, and these changes were prevented when hearts were pre-perfused with cyclosporin A prior to induction of ischemia. Exogenous cytochrome *c* stimulated the respiratory rate of skinned fibers from 30 min ischemic hearts to the control level and in fibers from 60 min ischemic hearts by about 35%. However, in fibers prepared from ischemic hearts pre-loaded with cy-

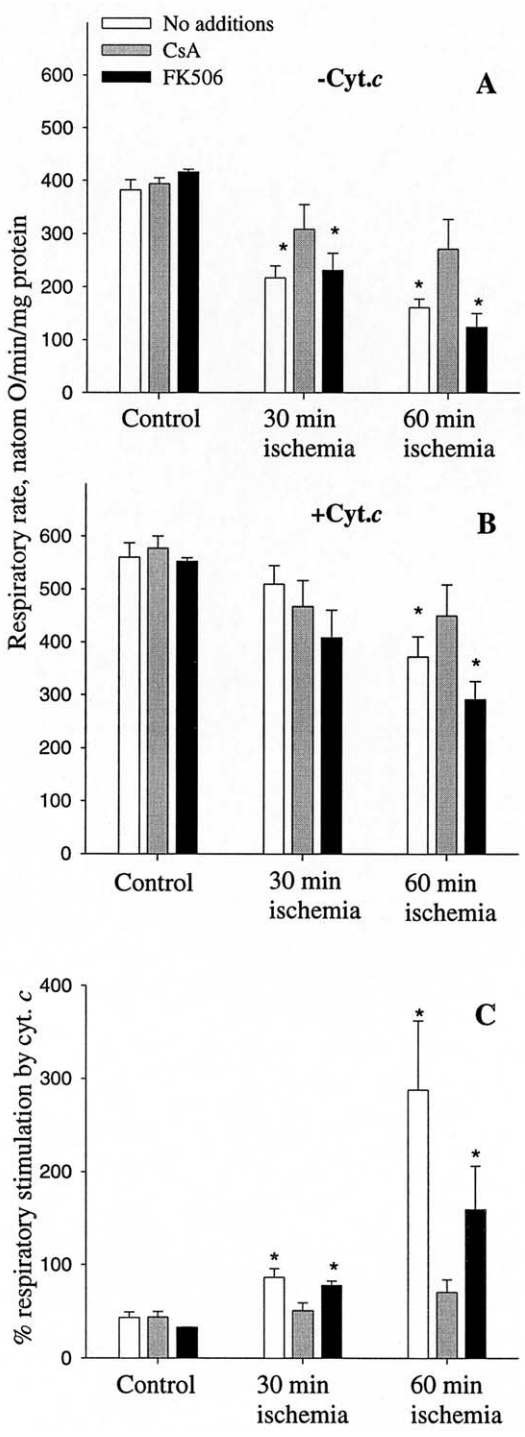

Fig. 3. Effect of exogenous cytochrome *c* on state 3 respiration rate in mitochondria isolated from control and ischemic hearts treated with cyclosporin A or FK506. Pyruvate 1 mM and malate 1 mM were used as respiratory substrates: (A) state 3 respiration rate without added cytochrome *c*; (B) state 3 respiration rate in the presence of 30 μM cytochrome *c*; (C) percentage stimulation by 30 μM cytochrome *c* of state 3 respiration rate in mitochondria from control and ischemic hearts. State 3 respiration rate before adding cytochrome *c* (during oxygen consumption measurements) was taken as 100%. Where indicated, hearts were pre-perfused with 0.1 μM cyclosporin A or 25 μM FK506 prior to inducing ischemia.

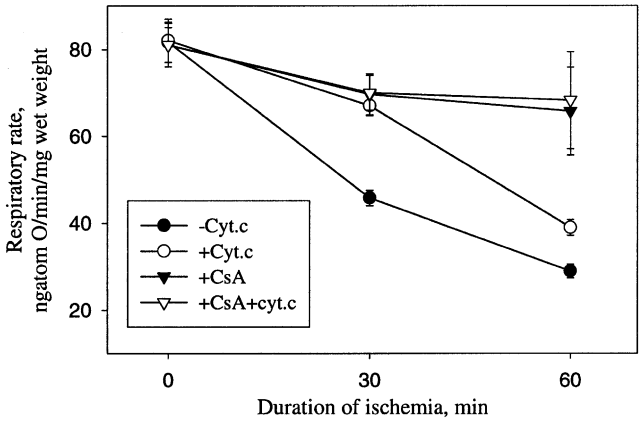

Fig. 4. The effect of exogenous cytochrome *c* on state 3 respiration rate in skinned fibers isolated from control and ischemic hearts treated with or without cyclosporin A. Skinned fibers were incubated in the medium B (see Section 2). 1 mM pyruvate + 1 mM malate were used as respiratory substrates. State 3 respiration rate was achieved adding 1 mM ADP followed by addition of 60 μM cytochrome *c*.

closporin A, there was no significant decrease in respiration rate after 30 min, nor after 60 min ischemia, and the effect of exogenous cytochrome *c* was reduced to 1–5% (the stimulation of control fibers was about 1%). Cyclosporin A also completely prevented loss of cytochrome *c*: the content of cytochrome *c* in control, 60 min ischemic and cyclosporin-treated 60 min ischemic fibers was 0.182 ± 0.015, 0.077 ± 0.003 and 0.186 ± 0.030 nmol/mg protein, respectively.

The data demonstrate that loss of cytochrome *c* from mitochondria during ischemia causes impairment of mitochondrial respiratory function, and both the loss of cytochrome *c* and mitochondrial dysfunction can be prevented by cyclosporin A, indicating that MPT is responsible for ischemic damage to the mitochondrial respiratory chain.

3.3. Ischemia-induced release of cytochrome c *from mitochondria is only partially caspase dependent*

Cytochrome *c* release from mitochondria could occur through caspase-dependent or -independent pathways. To investigate which pathway was involved during ischemia, we pre-perfused hearts with a cell-permeable inhibitor of caspases, DEVD-CHO, before inducing ischemia. The caspase inhibitor did not significantly prevent release of cytochrome *c* during 30 min ischemia (mitochondrial cytochrome *c* content decreased by 25% and 32% in DEVD-CHO-treated and -untreated hearts, respectively), though it significantly reduced the loss of cytochrome *c* after 60 min ischemia (32% and 50% in DEVD-CHO-treated and -untreated hearts, respectively; Fig. 5). The ischemia-induced inhibition of respiratory chain activity in mitochondria from DEVD-CHO treated hearts (Fig. 6) was similar to that in untreated hearts (compare to Fig. 2). Thus, cytochrome *c* release from mitochondria in this model of heart ischemia is largely caspase-independent, at least during the initial 30 min of ischemia. After longer times of ischemia caspase activation may facilitate the release of cytochrome *c*.

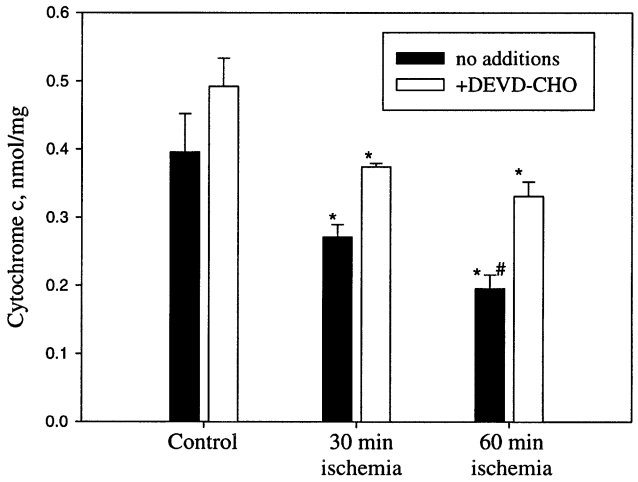

Fig. 5. Effect of caspase-inhibitor DEVD-CHO on cytochrome *c* content in control and ischemia-damaged mitochondria. Hearts were pre-perfused for 15 min with 1 μM DEVD-CHO, then ischemia was induced. Cytochrome *c* content in isolated mitochondria was determined spectrophotometrically. *—Statistically significant effect of ischemia ($P < 0.01$), if compared to control with the same treatment (with/without DEVD-CHO); #—statistically significant effect ($P < 0.05$), if compared to 30 min ischemia (without DEVD-CHO).

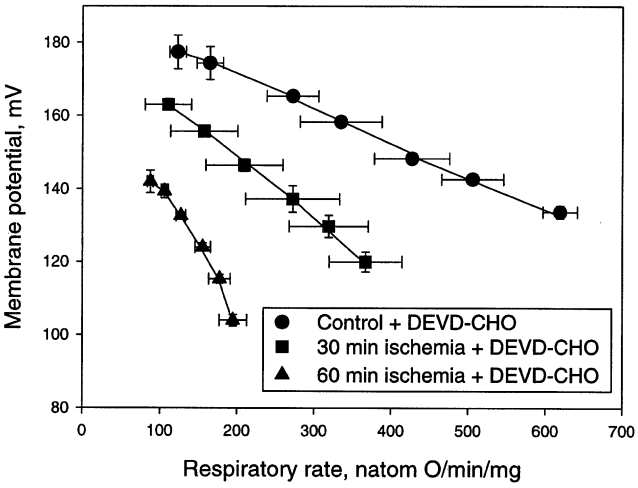

Fig. 6. Caspase-inhibitor DEVD-CHO does not prevent ischemia-induced inactivation of the mitochondrial respiratory chain. Membrane potential and rate of oxygen consumption of mitochondria isolated from hearts pre-perfused with 1 μM DEVD-CHO was measured as in Fig. 2. Succinate of 10 mM was used as the respiratory substrate in the presence of 1 μM rotenone.

3.4. Cyclosporin A prevents ischemia-induced caspase activation and nuclear fragmentation

Caspase activation is a characteristic feature of apoptosis. To determine the relation between ischemia-induced release of cytochrome *c* and induction of apoptosis, we investigated whether caspases were activated during ischemia and, if so, whether the activation can be prevented by cyclosporin A. Caspase activity in cytosolic extracts was measured using the synthetic peptide-substrate DEVD-pNA , which is mainly cleaved by caspase-3; however, other caspases, in particular, caspase-4, -6 and -7 can also contribute. Caspase-3-like,

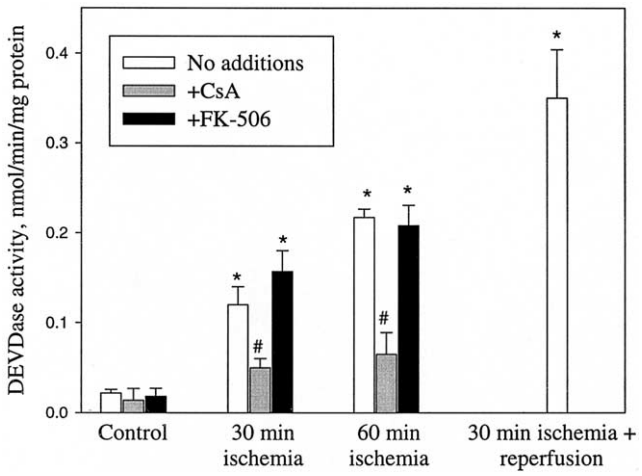

Fig. 7. Effects of cyclosporin A and FK506 on caspase activation during ischemia. Hearts were pre-perfused with 0.1 μM cyclosporin A, 25 μM FK506 or just with buffer prior to ischemia. DEVDase activity in heart cytosolic extracts was determined by measuring the DEVD-CHO-sensitive increase in absorption of p-nitroanillide formed during cleavage of DEVD-pNA. *The last bar in the figure represents caspase activity after 30 min ischemia + 60 min reperfusion (n = 5).* *—Statistically significant effect of ischemia *or ischemia/reperfusion* ($P < 0.01$), if compared to non-ischemic control of the same treatment; #—statistically significant effect of cyclosporin A ($P < 0.01$), if compared to treatment without cyclosporin A in the same group (30 or 60 min ischemia).

DEVD-cleaving activity was significantly increased after 30 min of ischemia (Fig. 7) and was further increased after 60 min ischemia. This correlated with the time-course of cytochrome *c* release from mitochondria during ischemia (see Fig. 1). Caspase activation was effectively (though not completely) blocked by cyclosporin A, but was insensitive to FK506 (Fig. 7) suggesting that it was mediated mainly by MPT-induced release of cytochrome *c*, but not by activation of calcineurin.

Caspase activation was followed by appearance of TUNEL-positive cardiomyocytes (Fig. 8). In the TUNEL assay, cells with DNA strand breaks are determined, which is another hallmark of apoptosis. No myocytes with DNA strand breaks were observed in sections from control hearts (Fig. 8). After 30 min ischemia, only very few (0.05%) TUNEL-positive myocytes were detected; however, the number of such cells greatly increased after 60 min ischemia, reaching 2%. Loading of hearts with cyclosporin A substantially reduced (to 0.03%) the number of TUNEL-positive cardiomyocytes after 60 min ischemia, which is consistent with reduced activity of caspases due to prevention of cytochrome *c* release from mitochondria after such treatment. The appearance of TUNEL-positive cells was also completely prevented when hearts were treated with the caspase-inhibitor DEVD-CHO prior to ischemia. This demonstrates that DNA strand breaks were indeed caused by caspases rather than non-specific, necrotic DNA damage.

We also tested whether reperfusion of the heart could cause a further increase in caspase activation or nuclear apoptosis. After 30 min ischemia, hearts were reperfused for 60 min. Reperfusion caused a further rise in caspase activity:

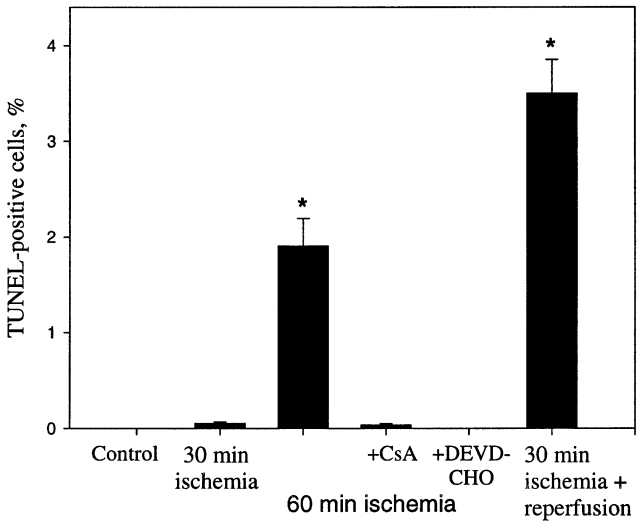

Fig. 8. Effects of cyclosporin A and caspase-inhibitor DEVD-CHO on ischemia-induced apoptosis in the heart assayed by TUNEL staining. The number of TUNEL-positive cardiomyocyte nuclei as a percentage of total number of cardiomyocytes in sections analyzed is presented. Only cells with clear myocyte morphology were counted. Total number of myocytes analyzed varied from 3000 to 10,000 in each group. *The last bar in the figure represents data for 30 min ischemia + 60 min reperfusion (n = 3).* *—$P < 0.01$ compared to control.

0.350 ± 0.054 nmol/min/mg after 60 min reperfusion vs. 0.108 ± 0.011 nmol/min/mg after 30 min ischemia alone, or 0.022 ± 0.005 nmol/min/mg in the non-ischemic control (Fig. 7). Reperfusion also caused an increase in the number of TUNEL-positive nuclei from 0.051 ± 0.013% after 30 min ischemia, 1.905 ± 0.291% at 60 min ischemia, to 3.500 ± 0.353% after 30 min ischemia followed by 60 min reperfusion (Fig. 8).

4. Discussion

Ischemia alone (in the absence of reperfusion) caused substantial cytochrome *c* release from the mitochondria to the cytosol, without gross damage to the mitochondria. This cytochrome *c* release was largely prevented by pre-perfusion of the hearts with 0.1 μM cyclosporin A, a concentration known to block ischemia-induced MPT in heart [18,19]. Care must be taken when interpreting results with cyclosporin A as it can also inhibit calcineurin (Ca^{2+}/calmodulin-dependent protein phospatase). However, at 0.1 μM, cyclosporin A causes little or no inhibition of calcineurin activity in heart, whereas 25 μM FK506 (which specifically inhibits calcineurin) causes substantial inhibition of calcineurin activity in the heart [28]. Such concentrations (5–25 μM) of FK506 have previously been used on perfused heart to inhibit calcineurin [28–30]. We found that 25 μM FK506 did not prevent ischemia-induced cytochrome *c* release or the related respiratory inhibition and caspase activation. Thus, the ability of cyclosporin to prevent these ischemic effects is not due to inhibition of calcineurin, but rather due to inhibition of MPT. MPT can cause cytochrome *c* release from isolated mitochondria, due to swelling of the mitochondria and rupture of the outer membrane [11,17]. Whether MPT occurs during heart ischemia or reperfusion is still controversial: most of the studies using relatively short times (about 30 min) of ischemia + reperfusion indicate MPT opening at reperfusion [15,18,19]; however, other studies suggest that MPT occurs during longer periods of ischemia [31,32]. Our own indirect evidence suggests that MPT occurs during prolonged ischemia, and this is the cause of cytochrome *c* release. The release of cytochrome *c* alone does not implicate MPT, but rather the prevention of that release as well as the caspase activation and nuclear apoptosis by cyclosporin A implicates MPT.

Pre-perfusion of the hearts with cyclosporin A substantially inhibited, but did not completely prevent ischemia-induced cytochrome *c* release (75% inhibition at 30 min, 65% inhibition at 60 min ischemia) and caspase activation (70% inhibition at 30 min, 74% at 60 min). This incomplete inhibition might be due to an inability of cyclosporin A to completely inhibit MPT under conditions of high calcium and/or low ATP/ADP levels [19], or caspase activation might be partially caused by other means, such as changes in BH3-domain proteins levels/location or activation of other proteases (calpains were recently shown to be involved in apoptosis in ischemic/reperfused heart [33]). Note, however, that cytochrome *c* release and caspase activation were no longer statistically significant in the presence of cyclosporin A (Figs. 1 and 7) and TUNEL staining was essentially completely prevented by cyclosporin A (Fig. 8). Note also that the two possibilities, that MPT or BH3-domain proteins are responsible for cytochrome *c* release, are not mutually exclusive, as it has been suggested that BH3-domain proteins cause cytochrome *c* release in part by inducing MPT [34,35].

MPT could potentially be induced during the reoxygenation occurring during isolation of the mitochondrial and cytosolic fractions. However, this possibility was greatly reduced by including EGTA and cyclosporin A in the ice-cold isolation medium, conditions designed to prevent MPT [19]. We found that COX level was unchanged in cytosols from control, ischemic or ischemic + cyclosporin A hearts (Fig. 1C), indicating that there was no gross mitochondrial damage during ischemia or differential damage during the isolation procedure. This is in agreement with our previous data showing that citrate synthase activity, another indicator of mitochondrial intactness, was not different in control and 60 min ischemic mitochondrial or cytosolic fractions, suggesting that mitochondria are not disrupted by the ischemia or subsequent isolation procedure [7].

We and others have previously shown that ischemia induces a substantial inhibition of the mitochondrial respiratory chain [12,22,36–39]. With relatively short periods of ischemia (30 min), this respiratory inhibition can be largely reversed by adding exogenous cytochrome *c*, and thus is, probably, largely due to the ischemia-induced release of cytochrome *c* [22,36,39]. A longer period of ischemia results in further respiratory inhibition, partly due to further cyto-

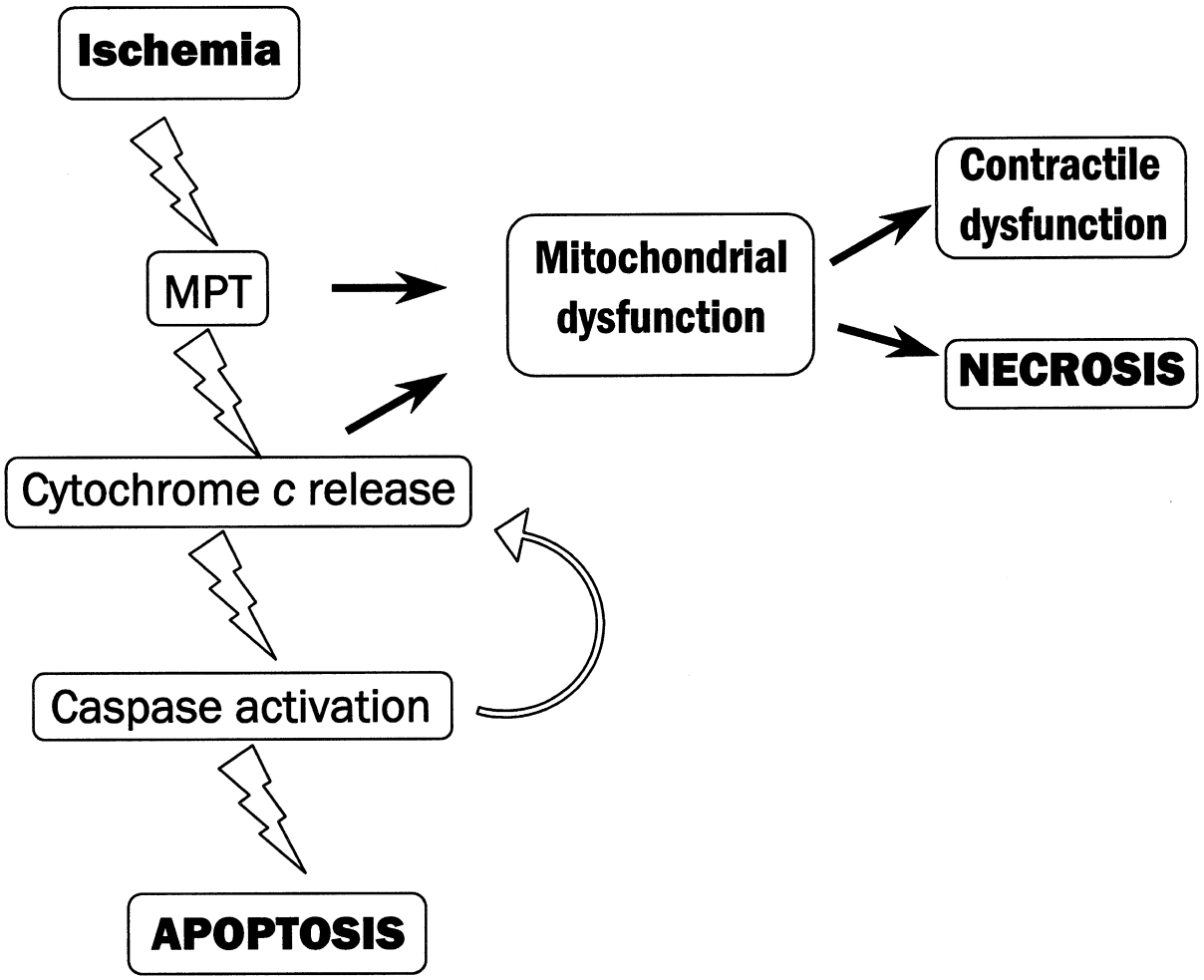

Fig. 9. Summary of factors mediating cell dysfunction and death in ischemic myocardium. Proposed scheme by which heart ischemia causes cell death and dysfunction. All arrows indicate a positive causal influence.

chrome *c* release, but also due to other mechanisms thought to include inhibition of complex I, the Krebs cycle, the ATP synthase and the adenine nucleotide translocator [36–40]. We found that the MPT inhibitor cyclosporin A (but not FK506) largely prevented the respiratory chain damage induced by 30 min ischemia, and partly prevented the damage induced by 60 min ischemia. As the ischemic-control, but not cyclosporin-treated, mitochondria had lost their cytochrome *c*, and the addition of exogenous cytochrome *c* partially restored respiration to ischemic-control mitochondria but not to cyclosporin-treated mitochondria, it seems likely that cyclosporin protected the mitochondrial respiratory chain by preventing cytochrome *c* release (secondary to MPT); but after 60 min of ischemia, some respiratory damage occurs independent of cytochrome *c* release (and MPT). The respiratory chain damage induced by ischemia may contribute to the contractile dysfunction or necrosis occurring after reperfusion.

Ischemia-induced cytochrome *c* release might, in principle, be a cause or a consequence of caspase activation. We found that cyclosporin A largely prevented ischemia-induced cytochrome *c* release, caspase-3-like protease activation and TUNEL staining, whereas a caspase-3 inhibitor, which was able to totally prevent ischemia-induced TUNEL staining was only able to partially prevent cytochrome *c* release. This suggests that cytochrome *c* release and subsequent caspase-3 activation and apoptosis is a consequence of MPT, but that caspase activity may feedback to cause further cytochrome *c* release, as has been described in other models of apoptosis [41]. This implies that apoptosis is induced by a mitochondrial route rather than a non-mitochondrial route. Another important implication is that inhibitors of MPT are likely to be more effective than caspase inhibitors in preventing ischemic damage.

TUNEL staining, indicative of DNA strand breaks, jumped from 0.05% of myocytes at 30 min of ischemia to 2% at 60 min, indicating this is a relatively late event in ischemia. This staining was completely prevented by a caspase-3-type inhibitor, indicating that the DNA fragmentation was mediated by caspases. This suggests that the TUNEL staining is quantifying the percentage of cells in end-stage apoptosis. Ischemia-induced TUNEL staining was almost completely prevented by cyclosporin A suggesting that ischemia-induced nuclear apoptosis is triggered solely via MPT. As

nuclear apoptosis was only 2% at 60 min ischemia, this would appear insignificant in terms of damage to the heart. However, the caspase-activation induced by ischemia is likely to cause further apoptosis during reperfusion [42]. Indeed, we found that reperfusion stimulated both caspase activity and nuclear apoptosis. Previous published findings have been contradictory as to whether apoptosis is induced during ischemia or rather during reperfusion [1,2,6,41,43]. Zhao et al. [41] found that 7 h of heart ischemia in vivo (coronary occlusion in dogs) caused no significant DNA fragmentation or laddering, whereas 1 h ischemia followed by 6 h reperfusion caused both. In contrast, Black et al. [43] found that 45 min ischemia in vivo (coronary occlusion in rats) caused substantial caspase-3 proteolytic activation and significant DNA fragmentation and laddering, and the latter two changes increased with 1–3 h of reperfusion. Similarly, Freude et al. [42] found that 45–90 min ischemia of perfused dog heart induced caspase activation, while TUNEL staining of nuclei required reperfusion. In isolated myocytes, De Moissac et al. [6] found that 1 h hypoxia alone caused DNA fragmentation and proteolytic activation of caspase-3. Reasons for these contradictory findings are unclear, but may relate to the degree of anoxia in different models of ischemia, age of experimental animals or species specificities.

The mechanism by which caspases are activated and apoptosis induced has also previously been investigated, but with little consensus. Ischemia has been known to cause cytochrome *c* release from mitochondria for decades (see Ref. [21] for review) and addition of cytochrome *c* to non-ischemic heart cytosol causes rapid caspase activation [7], presumably by binding to APAF-1 and activating pro-caspase-9. Thus, one possible cause of ischemic caspase activation is cytochrome *c* release from mitochondria. De Moissac et al. [6] found that 1 h of hypoxia caused cytochrome *c* release and this was not prevented by a caspase-3-type inhibitor (Ac-DEVD-CHO), but was partially prevented by a caspase-1-type inhibitor (Ac-YVAD-CHO). They tentatively suggested that caspase-1 might be upstream of cytochrome *c* release. However, Bialik et al. [2] found that glucose deprivation of isolated cardiomyocytes caused DNA fragmentation, DNA laddering, caspase-9 and caspase-3 proteolytic activation and cytochrome *c* release, but all these changes except cytochrome *c* release were prevented by a non-specific caspase inhibitor (zVAD-fmk), suggesting that caspase activation was all downstream of cytochrome *c* release. Our own results support this, but suggest that caspase activation may feedback to cause further cytochrome *c* release. The suggested scheme of events is sumMarized in Fig. 9: ischemia (without reperfusion) causes MPT and this induces cytochrome *c* release, which then causes caspase activation and apoptosis. Caspase activation may feedback to cause further cytochrome *c* release, and the cytochrome *c* release is partially responsible for mitochondrial respiratory dysfunction, which might contribute to contractile dysfunction or necrosis at reperfusion.

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