Minireview

Mitochondria in apoptosis of ischemic heart

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Abstract Apoptosis in the heart can be triggered by ischemia and/or reperfusion depending on conditions. This may involve activation of plasma membrane death receptors and/or translocation of Bcl-2 homologous proteins to mitochondria. However, one of the main mechanisms for triggering this apoptosis appears to be mitochondrial permeability transition followed by cytochrome *c* release. Cytochrome *c* release can result in caspase activation and thus apoptosis, but also results in mitochondrial dysfunction, which might contribute to contractile dysfunction or necrosis at reperfusion.

Key words: Apoptosis; Heart ischemia; Mitochondrion; Cytochrome *c*

1. Introduction

Apoptosis is a highly regulated form of cell death, essential to the normal development of an organism, and designed to eliminate unwanted cells. However, excessive apoptosis may contribute to pathological processes. The mechanisms of apoptotic cell death have been well studied in various non-myocytic cells and cell lines. In contrast, terminally differentiated cardiomyocytes have been considered to die exclusively by necrosis. Accumulating evidence indicates that cardiomyocyte apoptosis does occur in different cardiac diseases such as ischemic heart disease, heart failure and aging. Ischemia/reperfusion-induced cardiomyocyte apoptosis has been shown both in animals [1–4] and in humans [5–7].

There are a variety of endogenous inhibitors of apoptosis, some of which are exclusively expressed in myogenic cells (e.g. apoptosis repressor with caspase recruitment domain (ARC)) [8]. For example, IAP (inhibitor of apoptosis proteins), FLIP (FADD-like inhibitory proteins), some heat shock proteins and ARC inhibit apoptosis by binding and inhibiting caspases. The abundance of ARC and other inhibitory proteins in myocytes may be one possible explanation why apoptosis though present in ischemic/reperfused heart usually is not extensive (just a few percent of the total number of cardiomyocytes) compared to necrotic cell death [8]. However, in some conditions apoptosis is greater and even more abundant than necrosis [9,10]. Furthermore, apoptosis or its underlying mechanism – caspase activation – may subsequently cause necrosis, so that caspase inhibitors have been shown to reduce infarct size after ischemia [11,12].

Induction of apoptosis in ischemic/reperfused hearts has been suggested to be mediated by a variety of pathways which may or may not be interrelated, including: (a) activation of Fas or tumor necrosis factor-α (TNFα) receptors; (b) activation of p53 and c-Jun kinase pathways; (c) downregulation of antiapoptotic Bcl-2 protein and upregulation of proapoptotic Bax protein; (d) infiltration and activation of neutrophils and/or macrophages (reviewed in [13,14]). These pathways are more likely to be involved during reperfusion, whereas ischemia itself may initiate apoptosis via a mitochondrial pathway. Mitochondria play several roles in apoptosis: (a) they supply ATP that is necessary for execution of apoptosis; (b) they release cytochrome *c* and apoptosis-inducing factor proteins that are involved in caspase activation and nuclear fragmentation; (c) they release proteins (second mitochondria-derived activator of caspases SMAC/DIABLO) that neutralize endogenous inhibitors of apoptosis. In this short review we highlight the role of the mitochondrial pathway of apoptosis in ischemic heart and consider the consequences of mitochondrial cytochrome *c* loss.

2. When does apoptosis occur – during ischemia or reperfusion?

Previous published findings have been contradictory as to whether apoptosis is induced during ischemia or reperfusion. Apoptosis as an early and predominant form of cell death has been detected in human acute myocardial infarcts and it was shown to increase in reperfused myocardium [7]. In various animal models of ischemia/reperfusion it was shown that prolonged (6–7 h) ischemia causes no significant apoptosis but it is greatly accelerated by reperfusion [1,4,13]. Other investigators [2,3] found the first signs of apoptosis as early as after 10 min ischemia reaching maximum levels at 30–60 min ischemia with further increase [2] or no change [3] at reperfusion. Our own studies showed that caspase activation is already detectable after 30 min ischemia with DNA fragmentation at 60 min ischemia of isolated rat heart, and both parameters increased during reperfusion [15,16]. The reasons for these contradictory

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Abbreviations: ARC, apoptosis repressor with caspase recruitment domain; Apaf-1, apoptotic protease-activating factor; BH3, Bcl-2 homolog domain-2; ER, endoplasmic reticulum; MPT, mitochondrial permeability transition; TNFα, tumor necrosis factor-α; VDAC, voltage-dependent anion channel

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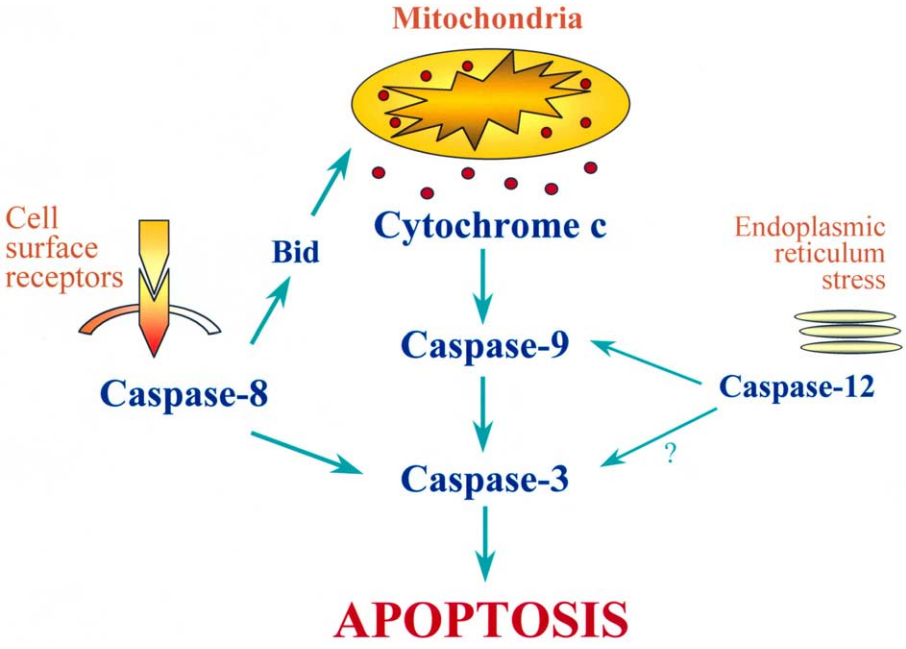

Fig. 1. Three pathways of caspase activation. In the receptor pathway, caspase-8 is recruited to a death receptor at the plasma membrane and then undergoes self-cleavage. The activated caspase-8 can then proteolytically activate caspase-3. Mitochondria release cytochrome *c*, which binds to the cytosolic protein Apaf-1 and facilitates activation of caspase-9. ER stress causes activation of caspase-12, which can activate caspase-9. There is cross-talk between the pathways, as caspase-8 can cleave cytosolic Bid, which can then bind to mitochondria and induce the release of cytochrome *c*.

findings are unclear, but may relate to the degree of anoxia in different models of ischemia, age of experimental animals or species specificities. There may be several phases of apoptosis following reperfusion, including an initial phase following rapidly after reperfusion, an intermediate phase following neutrophil infiltration into the heart, and a delayed phase days, weeks or months after the insult that may be involved in remodeling and heart failure [13]. Ischemia itself may vary in severity, ranging from acute total ischemia to chronic partial ischemia. The mechanisms of cell death involved in all these different phases and conditions are unlikely to be the same.

3. Which pathway causes caspase activation after ischemia?

Several of the morphological features of apoptosis are caused by caspase activity, and activation of caspases is often considered a key biochemical hallmark of apoptosis. Caspases are specialized cysteine-dependent proteases that cleave major structural proteins of the cytoplasm and nucleus [17]. In nor-

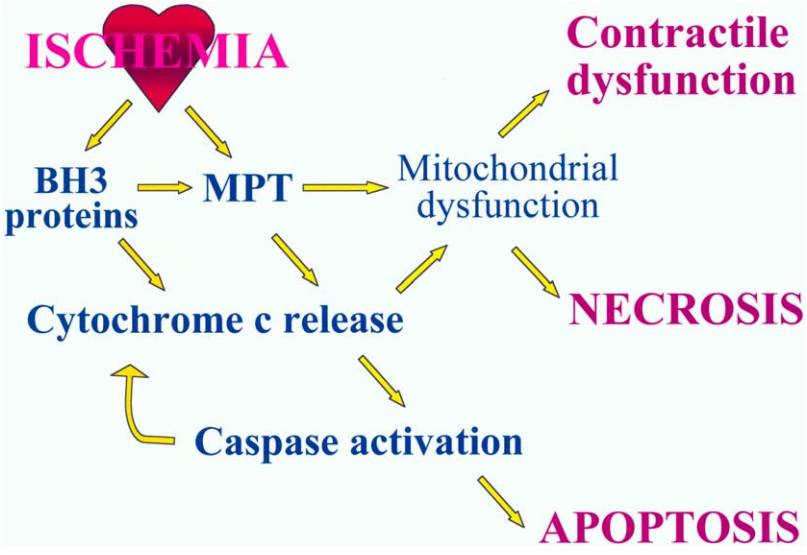

Fig. 2. Mitochondrial mechanisms of cell dysfunction and death in ischemic myocardium. Ischemia causes MPT-related cytochrome *c* release, which then induces caspase activation and apoptosis. Caspase activation may feedback to cause further cytochrome *c* release, and cytochrome *c* release is partially responsible for mitochondrial dysfunction, which might lead to contractile dysfunction or necrosis at reperfusion.

mal healthy cells caspases are present as poorly active or inactive zymogens that can be activated by autocleavage or processing by other proteases. To date three main pathways of caspase activation have been described (Fig. 1). The first pathway is initiated by binding of extracellular ligands (Fas ligand, TNFα) to their cell surface receptors leading to caspase-8 activation [18]. The second is mediated by mitochondria, which release apoptogenic proteins, mainly cytochrome *c*. Cytochrome *c* once released into the cytosol binds to a cytosolic protein apoptotic protease-activating factor (Apaf-1) and this complex in the presence of dATP or ATP facilitates activation of caspase-9, which in turn activates downstream caspase-3 [19,20]. The third pathway is initiated by endoplasmic reticulum (ER) stress (due to depletion of ER calcium or accumulation of unfolded proteins) and causes activation of caspase-12 [21], which recently has been shown to activate caspase-9 [22]. It remains unclear whether this pathway may operate in human cells because a human homologue of murine caspase-12 has not been found.

In principle, one or more of these pathways might be induced in ischemic/reperfused heart. Cardiomyocytes have Fas and TNFα receptors, and heart cells are known to produce Fas ligand and TNFα, which may activate the death receptor-mediated pathway [23,24]. Fas and TNFα have been implicated in late apoptosis after reperfusion: mice lacking functional Fas have reduced apoptosis 24 h after 30 min ischemia [25]. However, Fas and TNFα have not been implicated in apoptosis induced by hypoxia alone. During ischemia/reperfusion, there is also an increase in intracellular Ca^{2+} [26], which may lead to mitochondrial permeability transition (MPT) or ER stress. However, there is as yet no evidence that caspase-12 or ER stress is involved in apoptosis induced by heart ischemia/reperfusion.

In contrast to the other two potential pathways of caspase activation, there is strong evidence that the mitochondrial cytochrome *c* pathway participates in apoptosis induced by heart ischemia. Cytochrome *c* is released from mitochondria after 30–60 min of heart ischemia [15,16,27,28]; agents blocking cytochrome *c* release block apoptosis [16]; and addition of exogenous cytochrome *c* to heart cytosol (plus dATP) is sufficient to activate caspases to a similar extent as apoptosis [15].

4. What is the mechanisms of mitochondrial cytochrome *c* release?

Two mechanisms of cytochrome *c* release from mitochondria have been described. The first mechanism involves formation of a large channel in the outer mitochondrial membrane, due to oligomerization of certain Bcl-2 homologous proteins such as Bax or Bak. Bax is normally present as a monomer in the cytosol of many cells, including cardiomyocytes, but translocates to mitochondria and oligomerizes to form a pore in response to the translocation of Bcl-2 homologous domain-2 (BH3)-only proteins such as Bid, Bad and Bim [29–31]. Bak is normally present in mitochondria; Bax, Bid and Bad are cytosolic proteins but they translocate to mitochondria when apoptosis is induced [30,32]. Bid can be cleaved by caspase-8 and calpains, and then truncated Bid translocates to mitochondria causing cytochrome *c* release [31,33]. Bad can be dephosphorylated by calcium-dependent phosphatase calcineurin and then it translocates to mitochondria [34]. Recently a new Bcl-2 homologue, BNIP3, was found to be induced in cardiomyocytes by chronic hypoxia, while simultaneous acidosis was required for BNIP3 translocation to mitochondria and subsequent apoptosis, which appear to involve MPT but not caspases [35].

The second mechanism for cytochrome *c* release from mitochondria is mediated by opening of the MPT pore. MPT is a dramatic increase in permeability of the inner mitochondrial membrane to small (up to 1.5 kDa) molecules (for reviews see [36–38]). The MPT pore is formed at the site of contact between mitochondrial inner and outer membranes, and may consist of cyclophilin D, adenine nucleotide translocator, mitochondrial voltage-dependent anion channel (VDAC, also called porin), creatine kinase and possibly other proteins [36–38]. MPT can be induced by high calcium or oxidative stress especially when this is accompanied by depletion of adenine nucleotides, increase in phosphate levels and decrease in mitochondrial membrane potential, i.e. conditions present during heart ischemia and reperfusion. How MPT causes cytochrome *c* release is not clear yet. MPT causes mitochondrial swelling which may result in rupture of the mitochondrial outer membrane. However, mitochondrial swelling does not always occur in apoptotic cells, and studies on isolated heart or brain mitochondria have shown that release of cytochrome *c* can be induced by calcium or other MPT inducers without swelling [39,40]. BH3 proteins may also be involved in MPT pore formation. Bax-induced cytochrome *c* release in some cells or isolated mitochondria has been shown to be partially sensitive to cyclosporin A, suggesting involvement of MPT [41]. Bax, Bak and Bim have been shown to directly interact with VDAC and to induce cytochrome *c* release by accelerating opening of VDAC [42]. However, truncated Bid, Bim or Bax can cause cytochrome *c* release independently from MPT [43], probably by directly forming pores in the membrane phospholipid bilayers [44].

5. Does MPT occur during ischemia?

The most common approach to demonstrate the involvement of MPT in cell death is the use of cyclosporin A as a specific inhibitor of MPT. However, this must be taken with some caution because cyclosporin A also inhibits calcineurin, a calcium-dependent phosphatase, which besides many other roles in the cells can cause dephosphorylation of Bad and its translocation to mitochondria causing cytochrome *c* release. Using cyclosporin A MPT has been implicated in ischemia and reperfusion-induced damage to isolated hearts [45–48], or loss of heart tissue viability due to depletion of mitochondrial and cytosolic NAD^+ during reperfusion [49]. These studies concluded that MPT occurs during reperfusion rather than during ischemia itself. However, another study showed that cyclosporin protects from progression of cell injury during substrate-free anoxia of isolated cardiomyocytes [45], suggesting that MPT could occur during ischemia. Also, our own work showed that cyclosporin A but not the calcineurin inhibitor FK506 prevented ischemia-induced cytochrome *c* release from mitochondria, mitochondrial dysfunction, caspase activation and nuclear fragmentation in perfused rat heart [16].

Griffiths and Halestrap [47] developed a more direct method of measurement of MPT by perfusing hearts with 2-deoxy[^{3}H]glucose and then measuring how much had penetrated

into subsequently isolated mitochondria. They found that the label did not penetrate into mitochondria during ischemia, but did after reperfusion, suggesting that MPT occurred only at reperfusion. However, the interpretation of these experiments is complex, as it was found that perfusion of the hearts with cyclosporin A prior to ischemia did not decrease radiolabel uptake but rather increased it. The same study found that 30 min ischemia caused the yield (measured by citrate synthase activity) of subsequently isolated mitochondria to decrease by 33%, and this large loss of mitochondria was prevented by perfusion with cyclosporin A. This suggests that ischemia without reperfusion causes MPT-mediated damage to a proportion of the mitochondria, which may include damage to the inner membrane such that the radiolabel cannot be retained. Thus, it would appear that under some conditions MPT can occur during both ischemia and reperfusion.

The factors triggering MPT during ischemia/reperfusion are unclear, but have been suggested to include increased reactive oxygen/nitrogen species, calcium, phosphate, free fatty acids or decreased adenine nucleotides and mitochondrial membrane potential (reviewed in [37,38]).

6. What are the consequences of mitochondrial cytochrome *c* release during heart ischemia?

Cytochrome *c* release during heart ischemia may have a number of consequences: (1) caspase activation, (2) inactivation of the mitochondrial respiratory chain, and (3) mitochondrial free radical production. The cytochrome *c* release may have little or no consequence if the heart stays ischemic, as the ATP depletion will limit caspase activation and cause widespread necrosis. But if the heart is reperfused, the cytochrome *c* release and caspase activation that occurred during the previous ischemic period will trigger further caspase activation at reperfusion, potentially causing widespread apoptosis. This is consistent with our own and others' findings that reperfusion stimulates further caspase activation [16,50].

Release of cytochrome *c* from mitochondria inactivates mitochondrial respiration as cytochrome *c* is an essential component of the respiratory chain. Mitochondria isolated from ischemic heart have a severely inhibited respiratory chain, resulting in inhibited respiration and ATP synthesis [26,28,51]. And this inactivation of the respiratory chain, at least with short-term ischemia (30 min), is largely due to the loss of cytochrome *c*, as the inhibition can be reversed by simply adding cytochrome *c* to the isolated mitochondria [28]. With longer-term ischemia (45–60 min) mitochondrial dysfunction is also due to inhibition of complex I and the adenine nucleotide carrier, and due to uncoupling by fatty acids [26,28,52,53]. At reperfusion the inhibition of mitochondrial respiration and ATP synthesis resulting from ischemia may potentially contribute to the known contractile dysfunction, and necrosis. There is no direct evidence for this, but it is known that ischemia severely inhibits the respiratory chain, and that specific respiratory inhibitors can cause rapid contractile dysfunction and necrosis [26].

Loss of mitochondrial cytochrome *c* may potentiate mitochondrial production of oxidants at reperfusion, because (a) cytochrome *c* is a catalytic scavenger for mitochondrial superoxide, and (b) loss of cytochrome *c* results in reduction of complexes I, II and III potentiating superoxide production [54,55]. Thus loss of cytochrome *c* may contribute to the known burst of oxidant production when oxygen is returned at reperfusion. However, there is no direct evidence for ischemic loss of cytochrome *c* being responsible for the oxidant burst at reperfusion.

Note if MPT occurs during ischemia this will also promote necrosis by causing ATP depletion, via allowing ATP directly into the matrix and ATP hydrolysis by reversal of the ATP synthase.

7. Conclusions

Heart apoptosis can be triggered by ischemia and/or reperfusion depending on conditions. One of the main mechanisms for triggering this apoptosis appears to be MPT followed by cytochrome *c* release. Cytochrome *c* release can result in caspase activation and thus apoptosis, but also results in mitochondrial dysfunction, which might contribute to contractile dysfunction or necrosis at reperfusion (Fig. 2).

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