Confocal microscopy of the mitochondrial permeability transition in necrotic and apoptotic cell death

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Abstract

Opening of a high-conductance pore in the mitochondrial inner membrane induces onset of the mitochondrial permeability transition (mPT). Cyclosporin A and trifluoperazine inhibit this pore and block necrotic cell death in oxidative stress, Ca²⁺ ionophore toxicity, Reye-related drug toxicity, pH-dependent ischaemia/reperfusion injury and other models of cell injury. Confocal microscopy directly visualizes the increased mitochondrial membrane permeability of the mPT from the movement of calcein from the cytosol into the matrix space. Pyridine nucleotide oxidation, increased mitochondrial Ca²⁺ and mitochondrial generation of reactive oxygen species (ROS) all contribute to the onset of the mPT in situ. Confocal microscopy also shows directly that the mPT is a critical link in apoptotic signalling by tumour necrosis factor-α at a point downstream of caspase 8 and upstream of caspase 3.

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Cyclosporin A blocks this mPT, preventing release of pro-apoptotic cytochrome c from mitochondria and subsequent apoptotic cell killing. Progression to necrosis or apoptosis after the mPT depends on the availability of ATP, which blocks necrosis but promotes the apoptotic programme. Given the pathophysiological importance of the mPT, development of agents to modulate the mPT represents an important new goal for pharmaceutical drug discovery.

**The mitochondrial permeability transition**

Less than a decade ago, the mitochondrial permeability transition (mPT) was little more than an obscure biochemical curiosity. Its origins go back to the 1970s when Hunter and Haworth described a reversible phenomenon by which mitochondria become freely permeable to low-molecular-mass solutes [1–4]. Ca\(^{2+}\), inorganic phosphate and numerous oxidant chemicals induce this increased permeability, whereas Mg\(^{2+}\), ADP and low pH prevent onset of the mPT. As a consequence of the mPT, mitochondria depolarize, uncouple, release intramitochondrial solutes and undergo large-amplitude mitochondrial swelling. To the puzzlement of most biochemists, Hunter and Haworth had discovered a highly efficient mechanism to stop mitochondria from performing their best known task — the synthesis of ATP.

Renewal of interest in the mPT was stimulated in the late 1980s by the discovery that the immunosuppressive cyclic endecapeptide, cyclosporin A, specifically blocks the mPT [5,6]. Saturable inhibition by cyclosporin A implied that a specific protein mediates the mPT. Soon, by patch clamping, a cyclosporin A-sensitive pore was identified that conducts solutes of molecular mass less than 1500 Da [7–9]. Conductance of this PT pore in the mitochondrial inner membrane is very high. Opening of a single PT pore is estimated to be sufficient to cause mitochondrial depolarization and swelling [10].

**Composition of the PT pore**

The molecular make-up of the PT pore remains uncertain. The PT pore seems to be comprised, at least in part, of the adenine nucleotide translocase (ANT) protein [11], and pore activity has been reconstituted by inserting purified ANT into black lipid bilayer membranes and liposomes [12,13]. Other mitochondrial proteins may also associate with ANT to form a pore complex, including cyclophilin D (a cyclosporin A-binding protein) in the matrix, creatine kinase in the intermembrane space, and porin and hexokinase in the outer membrane [12–14]. Many speculate that the PT pore spans the inner and outer membrane at Hackenbrock’s contact sites [15]. However, pore activity is reported from mitochondrial membranes from triple ANT-knock-out yeast strains, implying that the ANT is not an obligatory component of the pore complex [16]. An alternative proposal is that the PT pore is a component of the protein complex that translocates nucleus-encoded proteins into mitochondria [17].
Protection by cyclosporin A against cellular injury

Soon after the discovery that cyclosporin A inhibits the mPT, reports began to appear that cyclosporin A protects against cytotoxicity from various stresses, including oxidative stress, hypoxia/ischaemia and chemical toxicants [18–27]. These reports suggested the mPT was a common pathophysiological event contributing to cell killing from these different injuries. However, cyclosporin A has other effects. The cyclosporin A–cyclophilin complex inhibits calcineurin, a protein phosphatase, which leads to immunosuppression in T-cells [28]. Calcineurin and the cyclophilins are widely distributed. Thus, cytoprotection by cyclosporin A could be mediated by its effects on calcineurin. Moreover, the nature of the intracellular environment opposes onset of the mPT after cell injury. Free Mg²⁺, which is about 0.5 mM in the cytosol, strongly inhibits the mPT. Much Mg²⁺ is chelated by ATP, and free Mg²⁺ actually increases as ATP falls during cell injury [29]. In addition, ADP increases and pH decreases during injury, and both ADP and acidic pH oppose

Figure 1 Loading of calcein and TMRM into rat hepatocytes. Overnight-cultured rat hepatocytes were initially loaded with calcein-AM (upper panels), followed by TMRM (lower panels). Calcein loaded predominantly into the cytosol, leaving mitochondria as dark voids in the green fluorescence (upper left). Red fluorescence was negligible in cells loaded only with calcein (upper right). After loading TMRM, mitochondria showed bright red fluorescence (lower right) that corresponded exactly to the dark voids in the green fluorescence of calcein (lower left).

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onset of the mPT. Thus a real question was whether the mPT could occur in situ in response to injurious stresses.

**Laser-scanning confocal microscopy to visualize mitochondrial membrane permeability in living cells**

Laser-scanning confocal microscopy creates submicrometre optical slices that exclude light arising from other planes of focus (reviewed in [30]). The result is unprecedented three-dimensional resolution of subcellular structures within thick cells and tissues. The resolving power of confocal microscopy is well illustrated when cultured cells, like rat hepatocytes or adult rabbit cardiac myocytes, are incubated at 37°C with calcein acetoxymethyl ester (calcein-AM). This neutral ester diffuses across the plasma membrane. Once inside the cytosol, esterases hydrolyse calcein-AM to its pentavalent free-acid form, which traps and retains the fluorophore inside the cytosolic compartment. However, the fluorophore loads poorly into mitochondria, which are outlined by green calcein fluorescence as dark round voids of about 1 μm in diameter (Figure 1, upper left) [31]. Subsequent loading with tetramethylrhodamine methyl ester (TMRM), a cationic fluorophore that localizes to mitochondria in response to their highly negative mitochondrial membrane potential [32,33], labels the voids with red fluorescence (Figure 1, lower right). Thus, virtually every dark void is an individual mitochondrion, although other organelles, notably lysosomes, can also exclude calcein. Importantly, addition of TMRM
has no effect on the calcein image. Thus, quenching or other interaction between the two fluorophores is not responsible for the dark voids. The mitochondrial profiles in the calcein images exist for the simple reason that calcein (molecular mass 623 Da) is highly impermeable to the mitochondrial inner membrane.

During normal aerobic incubation, mitochondria of rat hepatocytes and adult rabbit cardiac myocytes exclude calcein fluorescence for 1 h or more. If PT pores open transiently in these cells, then calcein should equilibrate between the cytosol and the mitochondria over time, but this is not observed [31,34]. Thus, the PT pore appears to remain closed on virtually a continuous basis in the absence of cellular stress.

During many forms of cell injury, including exposure to hypoxia, ischaemia/reperfusion, oxidative stress, toxic chemicals and calcium ionophores, the PT pore opens abruptly, which causes cytosolic calcein fluores-

Figure 3 Onset of the mPT after simulated ischaemia/reperfusion.
Cultured rat hepatocytes were loaded with TMRM and calcein, as described in Figure 1, and incubated with propidium iodide (PI). Confocal fluorescence images were collected at the end of 4 h of anoxia at pH 7.4 (simulated ischaemia) and after 5, 20 and 25 min of washout with oxygenated buffer at pH 7.4 (simulated reperfusion). At the end of ischaemia, virtually all mitochondrial TMRM fluorescence was lost. After reperfusion, mitochondrial TMRM fluorescence recovered within 5 min in both cells in the field. After 20 min, one cell lost TMRM labelling and went on to lose viability, as indicated by loss of calcein fluorescence and nuclear labelling with PI after 25 min (arrow). The other cell in the field continued to accumulate TMRM and did not lose viability. The lower panels (green fluorescence) show that mitochondria excluded calcein at the end of ischaemia and during the first 5 min of reperfusion. After 20 min of reperfusion, calcein fluorescence filled the mitochondria of the hepatocyte that had lost TMRM fluorescence, indicating onset of the mPT. In the cell that retained TMRM, mitochondria continued to exclude cytosolic calcein. After Qian et al. [36].
Fluorescence to redistribute from the cytosol into mitochondria [31,35–38]. Simultaneously, mitochondria depolarize, and mitochondrial TMRM fluorescence is lost, as illustrated in Figure 2 for hepatocytes exposed to a toxic concentration of salicylate. Although the mPT always causes mitochondrial depolarization, mitochondrial depolarization does not always imply onset of the mPT. For example, at the end of 4 h of simulated ischaemia to rat hepatocytes, mitochondria are depolarized, but they continue to exclude calcein in the cytosol (Figure 3).

Reye’s syndrome and the mitochondrial PT

In Reye’s syndrome, high fever, vomiting, fulminant hepatic failure, encephalopathy and lethargy progressing to coma and death develop in children after a prodromal viral illness [39–41]. Electron microscopy shows large-amplitude swelling in liver and brain mitochondria [42]. Metabolic changes such as suppression of ureagenesis and β-oxidation also implicate mitochondrial injury. Epidemiological studies implicated aspirin usage in the pathogenesis of Reye’s syndrome [43–45], and the disease virtually disappeared after an advisory by the U.S. Surgeon General in 1982 warned against aspirin use in children [46]. Although Reye’s syndrome associated with aspirin usage has declined, a number of Reye-related illnesses have been identified in association with other drugs and substances [47–50]. The best known is that associated with the widely prescribed anticonvulsant, valproic acid. Ingestion of the unripe fruit of the ackee tree in Jamaica and exposure to Neem (Margosa) oil in India also produce a Reye-like illness. Reye-like disease can also develop in inherited metabolic disorders involving organic acid metabolism [51], and a microvesicular steatosis occurs idiospathically in pregnancy that is similar to Reye’s syndrome [52].

Mitochondrial dysfunction and large-amplitude swelling in Reye’s syndrome patients suggested to us that onset of the mPT might be the basis of the pathophysiology in Reye-related disease. In support of this idea, we found that salicylate induces a cyclosporin A-sensitive mPT in isolated rat liver mitochondria [53]. Although others reported that aspirin induces mitochondrial swelling and the mPT [54,55], we found that aspirin does not induce the mPT unless it is first hydrolysed to salicylate. Other chemicals implicated in Reye’s-related disorders also induce cyclosporin A-sensitive mitochondrial swelling, including Neem oil and adipic, isovaleric, 3-mercaptopropionic, 4-pentenoic and valproic acids [53]. Although salicylate is a weak uncoupler of mitochondrial oxidative phosphorylation, membrane depolarization is not the mechanism by which Reye’s-related chemicals promote the mPT [53].

Salicylate also induces the mPT and cell death in situ in cultured rat hepatocytes. This was demonstrated by confocal microscopy showing the redistribution of calcein from the cytosol into the mitochondria (Figure 2) [38]. After onset of the mPT, hepatocytes soon lose viability. Both the mPT and loss of cell viability after salicylate are blocked by cyclosporin A and its non-immunosuppressive analogue, 4-methylvaline cyclosporin. These data in
isolated liver mitochondria and cultured hepatocytes support the hypothesis that the mPT is involved in the pathophysiology of Reye’s syndrome.

In isolated mitochondria, induction of the mPT by salicylate does not occur in the absence of added Ca^{2+} [53]. Similarly, salicylate toxicity to cultured hepatocytes occurs more rapidly at higher extracellular Ca^{2+} concentrations. Consistent with this Ca^{2+} dependence, calcium antagonists such as the Ca^{2+} channel blocker, verapamil, decrease salicylate-induced killing of hepatocytes. Protection by verapamil is associated with decreased mitochondrial free Ca^{2+} determined by confocal microscopy of rhodamine-2, a Ca^{2+}-indicating fluorophore [38].

**The mitochondrial PT in the pH paradox of ischaemia/reperfusion injury**

In ischaemia, tissue pH decreases rapidly due to anaerobic glycolysis and the hydrolysis of ATP. Rather than aggravating injury, this naturally occurring acidosis actually protects strongly against loss of cell viability during ischaemia [56–59]. In sharp contrast, the restoration of normal pH after reperfusion of ischaemic tissue rapidly accelerates cell killing, a phenomenon we call the pH paradox [58,60–67]. In a variety of models of ischaemia/reperfusion with cells and tissues from liver and heart, reperfusion at an acidotic pH (e.g. pH 6.5) prevents cell killing almost entirely. Cell killing in the pH paradox does not depend on the formation of ROS, since anaerobic reperfusion at normal pH causes as much cell killing as reperfusion in the presence of oxygen.

Hydrogen ions appear to mediate cytoprotection directly. Acidic intracellular pH is cytoprotective even when intracellular and extracellular Na^{+} concentrations are equilibrated with monensin [59]. Similarly, pH-dependent cell killing is unrelated to concentrations of cytosolic and extracellular free Ca^{2+} [62,63]. Thus secondary changes of intracellular Na^{+} or Ca^{2+} do not mediate cell killing in the pH paradox.

Since pH<7 inhibits conductance through the PT pore, we investigated the hypothesis that onset of the mPT causes pH-dependent reperfusion cell killing in the pH paradox [36]. In support of the hypothesis, cyclosporin A prevents pH-dependent loss of cell viability after simulated ischaemia/reperfusion of cultured hepatocytes. Notably, cyclosporin A exerts its protective effect even when added only during the reperfusion phase, which underscores that cell killing is the specific consequence of reperfusion.

Confocal microscopy provided direct support for the hypothesis that the mPT mediates pH-dependent reperfusion injury [36]. In cultured rat hepatocytes, anoxia at pH 6.2 simulates ischaemia, and re-oxygenation at pH 7.4 simulates reperfusion. During simulated ischaemia in this model, onset of the mPT fails to occur even after 4 h of anoxia, since calcine fluorescence does not redistribute from the cytosol to the mitochondria (Figure 3). By contrast, mitochondrial depolarization does occur, since virtually all mitochondrial TMRM fluorescence is lost at the end of the ischaemic period. After re-oxygenation at normal pH (simulated reperfusion), mitochondria begin to repolarize. In most cells, however, calcine subsequently redistributes from the cytosol into the
mitochondria, and the accumulated TMRM is once again lost. This increased mitochondrial membrane permeability and depolarization demonstrates directly the onset of the mPT (Figure 3). In hepatocytes showing onset of the mPT, viability is lost afterwards, whereas viability is retained when the mPT does not occur. The mPT and cell killing are prevented when cells are reperfused in the presence of cyclosporin A or with acidotic buffer. Thus, the mPT is causing pH-dependent reperfusion injury in this model [36].

Contribution of the mPT to cell injury during oxidative stress

The short-chain analogue of lipid hydroperoxides, t-butylhydroperoxide (TBH), induces the mPT in isolated mitochondria. Similarly in rat hepatocytes, TBH induces onset of the mPT, mitochondrial depolarization and ATP depletion, culminating in cell death [25,31]. Trifluoperazine blocks the mPT in isolated mitochondria. In hepatocytes exposed to TBH, trifluoperazine also prevents calcein redistribution from the cytosol into mitochondria, loss of mitochondrial TMRM fluorescence, ATP depletion and cell death, supporting the conclusion that TBH-induced cell killing is mediated by onset of the mPT. Protection by trifluoperazine is specific for the mPT-mediated injury, since trifluoperazine does not prevent the cytotoxicity of protonophoric uncouplers that also produce mitochondrial depolarization, ATP depletion and cell death but not the mPT [31].

TBH induces oxidation of mitochondrial pyridine nucleotides through the concerted action of glutathione peroxidase, glutathione reductase and pyridine nucleotide transhydrogenase [68–70]. Oxidation of pyridine nucleotides can be visualized directly by UV confocal microscopy as a rapid decline of NAD(P)H autofluorescence in TBH-treated hepatocytes [71,72]. Oxidation of mitochondrial pyridine nucleotides is followed closely by an increase of mitochondrial free Ca²⁺, measured with rhodamine-2, and generation of ROS within mitochondria, measured by the ROS-dependent conversion of dichlorofluorescin to highly fluorescent dichlorofluorescein (Figure 4). Subsequently, onset of the mPT occurs, mitochondria depolarize and viability is lost.

Each of these changes contributes to the progression to cell death. β-Hydroxybutyrate, which increases mitochondrial NAD(P)H by the β-hydroxybutyrate dehydrogenase and transhydrogenase reactions, delays TBH-induced cell killing, indicating that mitochondrial pyridine nucleotide oxidation is important for initiating cell death. By contrast, lactate, which increases cytosolic NADH via the lactate dehydrogenase reaction, does not protect [71]. Thus the specific oxidation of mitochondrial pyridine nucleotides contributes to onset of the mPT and cell killing after oxidative stress, in agreement with observations in isolated mitochondria [73–75].

In hepatocytes exposed to TBH, mitochondrial ROS generation increases 15-fold, as determined by confocal microscopic measurements of dichlorofluorescein fluorescence (Figure 4) [71]. ROS production occurs after the initial rapid oxidation of NAD(P)H by TBH. Desferal, an iron chelator, and diphenylphenylenediamine, a free-radical scavenger, block ROS formation,
onset of the mPT and cell killing. Thus, ROS are another factor promoting onset of the mPT during oxidative stress with TBH. One mechanism of PT pore opening may be ROS-mediated oxidation of protein thiols, as shown by studies in isolated mitochondria [73,76]. Mitochondrial ROS are also formed during excitotoxic stress in neurons [77,78], which is consistent with several recent reports showing that the mPT mediates excitotoxic injury to neurons [79–83].

Contribution of mitochondrial free Ca\(^{2+}\) to mitochondrial ROS production and onset of the mPT

Intramitochondrial Ca\(^{2+}\) chelation with bis-(o-aminophenoxo)ethane-N,N,N',N'-tetra-acetic acid (BAPTA) AM prevents the rise of mitochondrial free Ca\(^{2+}\) after exposure of hepatocytes to TBH [72]. Ca\(^{2+}\) chelation also completely blocks the stimulation of mitochondrial ROS generation by TBH but does not prevent the early rapid oxidation of mitochondrial NAD(P)H. BAPTA AM, however, does inhibit the late phase of more complete oxidation of mitochondrial pyridine nucleotides that is attributed to ROS formation [71]. Overall, these results support a model in which the initial effect of TBH is
mitochondrial NAD(P)H oxidation (Figure 5). NAD(P)H oxidation disrupts mitochondrial Ca\(^{2+}\) homoeostasis and leads to a net increase of mitochondrial Ca\(^{2+}\). Increased mitochondrial Ca\(^{2+}\) then stimulates mitochondrial formation of ROS. Together, increased Ca\(^{2+}\) and ROS formation induce onset of the mPT, leading to collapse of the mitochondrial membrane potential (\(\Delta \Psi_m\)), ATP depletion and bioenergetic cell death. ROS formation feeds back to augment pyridine nucleotide oxidation and Ca\(^{2+}\) dysregulation, which further amplifies the signals promoting the mPT. \(\beta\)-Hydroxybutyrate (BHB) increases mitochondrial pyridine nucleotide reduction and delays cell injury. Intramitochondrial BAPTA prevents mitochondrial Ca\(^{2+}\) from increasing after TBH, an effect that blocks ROS formation, the mPT and subsequent cell death. Diphenylphenylenediamine (DPPD) and desferal also block ROS formation and prevent the mPT and cell killing. After Byrne et al. [72].

**Figure 5 Scheme of mitochondrial changes inducing onset of the mPT during oxidant stress with TBH.** TBH oxidizes mitochondrial pyridine nucleotides (NADH and NADPH), causing dysregulation of mitochondrial Ca\(^{2+}\) homoeostasis. Increased mitochondrial Ca\(^{2+}\) stimulates mitochondrial formation of ROS. Together, increased Ca\(^{2+}\) and ROS formation induce onset of the mPT, leading to collapse of the mitochondrial membrane potential (\(\Delta \Psi_m\)), ATP depletion and bioenergetic cell death. ROS formation feeds back to augment pyridine nucleotide oxidation and Ca\(^{2+}\) dysregulation, which further amplifies the signals promoting the mPT. \(\beta\)-Hydroxybutyrate (BHB) increases mitochondrial pyridine nucleotide reduction and delays cell injury. Intramitochondrial BAPTA prevents mitochondrial Ca\(^{2+}\) from increasing after TBH, an effect that blocks ROS formation, the mPT and subsequent cell death. Diphenylphenylenediamine (DPPD) and desferal also block ROS formation and prevent the mPT and cell killing. After Byrne et al. [72].
by increasing NAD(P)H oxidation. This feedback amplifies the signals promoting the mPT (Figure 5) [72].

**Involvement of the mPT in cell death by apoptosis**

Necrosis and apoptosis have long been considered fundamentally different processes. Necrotic cell death is the consequence of acute disruption of cellular metabolism, which leads to ATP depletion, ion dysregulation, mitochondrial and cellular swelling, activation of degradative enzymes and plasma membrane rupture [84]. In apoptosis, by contrast, metabolism is not severely impaired. In its purest form, apoptosis represents a special form of cellular differentiation that leads to the orderly resorption of target cells [85]. Apoptosis is mediated, in large part, by activation of a cascade of cysteine aspartate proteases called caspases.

Given these differences, it came as a surprise that the mPT is not only implicated in necrosis but in apoptosis as well. In a cell-free system combining purified mitochondria and nuclei, induction of the mPT causes mitochondria to release soluble factors that activate caspases [86]. One of these factors is cytochrome c [87]. Cytochrome c ordinarily resides in the intermembrane space as a diffusible electron carrier [88], but it is released into the cytosol when mitochondrial swelling induced by the mPT breaks the mitochondrial outer membrane.

The exact role of the mPT in mediating apoptosis is controversial, and some studies claim that cytochrome c release during apoptosis occurs without mitochondrial depolarization and by implication without the mPT [89,90]. However, in apoptosis induced by tumour necrosis factor-α (TNFα) in hepatocytes, onset of the mPT as visualized by confocal microscopy can be shown to precede cytochrome c release, activation of caspase-3, DNA degradation and the morphological changes of apoptosis [91]. Further, cyclosporin A inhibits the TNFα-induced mPT in hepatocytes and blocks cytochrome c release, caspase-3 activation and subsequent apoptosis. As hepatocytes undergo apoptosis in this model, onset of the mPT occurs progressively through the mitochondria of each cell. As visualized by loss of TMRM fluorescence and uptake of calcine fluorescence, initially just a few mitochondria undergo the mPT after about 7 h of TNFα exposure (Figure 6). Over the next few hours, the mPT occurs in the remainder of the mitochondria, and four or more hours can pass between onset of the mPT in the first and last mitochondria of a cell exposed to TNFα. Thus, for a period of time, normal polarized mitochondria co-exist with mitochondria that have undergone the mPT. This may explain reports in which cytochrome c release was observed in cells still containing polarized mitochondria.

**Metabolic consequences of mitochondrial injury**

The importance of mitochondrial dysfunction in cytotoxicity can be assessed by the ability of glycolytic substrates to rescue cells from lethal injury.
ATP generated from glycolysis prevents ATP depletion after mitochondrial injury and averts cell killing. Glucose and endogenous glycogen are excellent glycolytic substrates for most cells, but in hepatocytes glucose is poorly metabolized. Fructose is a much better substrate, and fructose protects hepatocytes against loss of viability after exposure to anoxia, cyanide, oligomycin and a variety of oxidant chemicals, suggesting that mitochondria are the common target of a wide variety of toxicants and toxic stresses [58,80,92–94]. ATP need not

Figure 6 Onset of the mPT during apoptosis induced by TNFα. Rat hepatocytes expressing the IκB super-repressor were treated with TNFα and loaded with calcein and TMRM, as described in Figure 1. After 7 h exposure to TNFα, little mitochondrial depolarization (loss of TMRM fluorescence, left panel) or increased mitochondrial permeability to calcein (right panel) was evident. Subsequently, the mPT occurred in individual mitochondria over the next 2 h. At 9 h, almost all mitochondria had depolarized and become permeable to calcein. In other experiments, an interval of four or more hours was needed for onset of the mPT to occur in all the mitochondria of a single hepatocyte. After Bradham et al. [91].
recover to normal for protection to occur. Indeed, only a small percentage of normal ATP, perhaps 10–20%, needs to be present to prevent cell killing.

When a toxicant causes uncoupling of mitochondrial oxidative phosphorylation, then glycolytic ATP generation does not protect against cell killing. Mitochondrial uncoupling activates the oligomycin-sensitive mitochondrial F1F0-ATPase, which hydrolyses ATP produced by glycolysis. When mitochondrial injury includes uncoupling, the combination of glycolytic substrate plus oligomycin protects against cell killing, even though oligomycin alone is toxic in the absence of glycolytic substrate [93,94]. Thus cytoprotection by oligomycin plus glycolytic substrate but not by either alone indicates that uncoupling of oxidative phosphorylation mediates cell death. By this criterion, activation of mitochondrial ATPase and consequent ATP depletion cause the toxicity not only of classical uncouplers but also of ionophores like Br-A23187 and monensin.

What determines whether apoptosis or necrosis develops after onset of the mPT?

If the mPT precedes both necrotic and apoptotic cell death, what determines how cell killing will occur? Since apoptosis stimulated by cytochrome c requires ATP (or dATP) [87], the presence or absence of ATP after onset of the mPT may be the determining factor in whether or not apoptosis develops. Several studies show that apoptosis can only develop when ATP is present [95–97]. Onset of the mPT causes mitochondrial uncoupling and inhibition of ATP production by oxidative phosphorylation. However, if glycolysis is pre-
sent to a substantial extent, then ATP production may persist even after the mPT. Thus, the existence of an alternative ATP source to oxidative phosphorylation may be a critical factor allowing cells to progress to apoptosis. Similarly, if onset of the mPT occurs in only a subpopulation of mitochondria, as during the early stages of TNF-α/H9251-induced apoptosis in hepatocytes, then the remaining functional mitochondria may produce sufficient ATP to permit apoptosis to proceed.

Experiments with the Ca²⁺ ionophore, Br-A23187, support this concept. Acute exposure of hepatocytes to Br-A23187 causes rapid necrotic cell death that is mediated by onset of the mPT and prevented by cyclosporin A [37,98]. Fructose plus oligomycin also prevent this necrotic cell killing by provision of glycolytic ATP and inhibition of the mitochondrial ATPase. However, fructose and oligomycin do not block onset of the mPT, as visualized directly by confocal microscopy. In the presence of fructose plus oligomycin, Br-A23187 now produces apoptosis instead of necrosis, and nuclear changes indicative of apoptosis become apparent after about 10 h (Figure 7). This apoptosis is still dependent on the mPT, since it is prevented by cyclosporin A [98]. Thus when the mPT depletes ATP, necrotic cell death occurs, but when onset of the mPT develops without ATP depletion, apoptosis ensues (Figure 8).

**Figure 8 Scheme showing the role of ATP in apoptotic and necrotic cell killing mediated by the mPT.** The mPT can induce both necrosis and apoptosis. When the mPT occurs abruptly, inhibition of oxidative phosphorylation and activation of mitochondrial ATPases causes ATP depletion, leading to necrotic cell death, as occurs when hepatocytes are exposed to the Ca²⁺ ionophore, Br-A23187. If the mitochondrial ATPase is inhibited with oligomycin and a glycolytic substrate is available, then ATP levels are preserved even after onset of the mPT. Under these conditions, apoptotic rather than necrotic cell death ensues. In both conditions, apoptotic and necrotic cell death are prevented by cyclosporin A. After Qian et al. [98].
Therapeutic implications

The importance of understanding pathophysiological mechanisms is that such understanding may lead to new therapeutic interventions. Much evidence now implicates the mPT in a wide range of disease states. If the mPT is the key pathophysiological event causing necrotic and apoptotic cell killing, then pharmacological antagonists of the mPT should have the potential to prevent or even reverse disease. For example, one recent study showed that cyclosporin A and trifluoperazine in combination with fructose protect against hepatotoxicity caused by acetaminophen in rats [99]. Unfortunately, both cyclosporin A and trifluoperazine have biological effects that make these drugs potentially unsuitable for very ill patients. Thus, an important goal for pharmaceutical research should be the discovery of additional, more specific, blockers of the mPT.

This work was supported, in part, by grants DK37034, AG07218 and AG13318 from the National Institutes of Health.

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