Mechanisms of Hepatic Toxicity
V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis*

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Lemasters, John J. Mechanisms of Hepatic Toxicity. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1–G6, 1999.—Opening of a high-conductance pore conducting solutes of molecular mass <1,500 Da causes onset of the mitochondrial permeability transition (MPT). Cyclosporin A blocks this pore and prevents acute necrotic cell death in several models. Confocal microscopy directly visualizes onset of the MPT during acute cytotoxicity from the movement of the green-fluorescing fluorophore, calcein, into the mitochondria from the cytosol. The MPT also plays a causative role in tumor necrosis factor-α-induced apoptosis in hepatocytes. Progression to apoptosis or necrosis after the MPT may depend on the presence or absence, respectively, of ATP. Often, features of both apoptotic and necrotic cell death develop after death signals and toxic stresses. The term "necrapoptosis" is introduced to emphasize the shared pathways leading to both forms of cell death.

ATP; confocal microscopy; cyclosporin A; tumor necrosis factor-α

UNTIL RECENTLY, THE mitochondrial permeability transition (MPT) was an obscure phenomenon associated with mitochondrial swelling and lysis when the organelles were treated somewhat harshly in vitro. Studied by only a handful of researchers until the late 1980s, the MPT was first characterized by Hunter et al. (14) as a reversible Ca²⁺-induced permeabilization of the mitochondrial inner membrane (reviewed in Refs. 2 and 40). Permeabilization in the MPT is selective for solutes of molecular mass less than ~1,500 Da. Single-channel recordings subsequently confirmed that opening of a nonspecific, high-conductance pore in the inner membrane precipitates the MPT (37). Conductance of this pore is so great that opening of only a few pores, possibly only one, is sufficient to cause mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large-amplitude mitochondrial swelling, the signature changes of the MPT (40).

The list of agents that promote onset of the MPT is long (see Ref. 10). Notably, Ca²⁺, which must be transported into mitochondria, inorganic phosphate, reactive oxygen species (ROS), and a variety of oxidant chemicals induce onset of the MPT. In addition, membrane depolarization and cross-linking of thiol in the pore complex promote pore conductance (1, 6). Other factors block onset of the MPT. These include Mg²⁺ (pH below ~7), a variety of phospholipase inhibitors (including bicine, mepacrine, and trifluoperazine), and the immunosuppressive cyclic endecapeptide, cyclosporin A. Indeed, saturable inhibition of the MPT by nanomolar concentrations of cyclosporin A removed any doubt that the MPT was caused by opening of a specific pore in the mitochondrial inner membrane rather than by a less specific perturbation of lipid bilayer organization.

Given the low abundance of the pore, it is not surprising that molecular characterization of the pore complex has progressed slowly. Observations that inhibitors of the adenine nucleotide translocator (ANT) either induce or inhibit the MPT led to the proposal that the ANT is an essential component of the permeability transition pore (11) and pore conductance has been reconstituted with purified ANT (5). Inhibition of pore conductance by cyclosporin A suggests that the cyclosporin A binding protein, cyclophilin D, found in the mitochondrial matrix is also a component of the pore complex. Other evidence suggests that the pore complex contains VDAC (voltage-gated anion channel), a protein in the outer membrane. These various components presumably come together at contact sites between the inner and outer membranes. Several partially purified preparations with pore conductances have been described that contain additional proteins with possible regulatory effects, including hexokinase, creatine kinase, and the proapoptotic protein, Bax (3, 26). However, not all evidence supports this model. One report claims to measure pore conductance from mitochondrial membranes of triple ANT knockout yeast strains, implying that the ANT is not an obligatory component of the pore complex (24), and another proposal is that the permeability transition pore is part of the import machinery that translocates nucleus-encoded proteins into mitochondria (23).

MPT IN ACUTE CELL INJURY

In 1990, soon after the discovery that cyclosporin A inhibits the MPT, the first report appeared that cyclosporin A blocks cell death after an injurious stress (15). Subsequently, a large number of reports showed cytoprotection by cyclosporin A against injury from oxidative stress, anoxia, ischemia-reperfusion, and a variety of toxic chemicals (reviewed in Ref. 21). However, cyclosporin A has other pharmacological effects. Its immunosuppressive action, which is independent of its effect on the MPT, acts by inhibition of calcineurin, a protein phosphatase involved in T cell activation (12). Thus calcineurin inhibition might be the basis for cytoprotection. Moreover, free Mg$^{2+}$ in the cytosol of normal cells is 0.5 mM or greater, a concentration that strongly inhibits the MPT in isolated mitochondria. Therefore, it remained possible that cytoprotection by cyclosporin might be unrelated to the MPT, and in situ documentation of onset of a cyclosporin A-sensitive MPT in cells during the progression of injury was necessary.

Direct observation of the MPT in situ became possible with use of the three-dimensional resolving power of laser scanning confocal microscopy (31). When calcein, a green-fluorescing dye, was ester loaded into the cytosol of cultured cells such as rat hepatocytes and rabbit cardiac myocytes, confocal microscopy revealed numerous dark round voids in the otherwise diffuse green fluorescence of the cytoplasm (Fig. 1). Each of these voids is a single mitochondrion, and the voids exist for the simple reason that the mitochondrial inner membrane is impermeable to calcein, an organic polyanion of 623 Da. In contrast, after exposure of hepatocytes to toxic stresses, including oxidant chemicals, ischemia-reperfusion, Reye-related drugs, and Ca$^{2+}$ ionophore, calcein abruptly redistributes from the cytosol into the mitochondria, causing the dark round voids to fill with fluorescence (see Ref. 21) (Fig. 1). Simultaneously, the mitochondria depolarize, as indicated by the release of membrane potential-indicating dyes like tetramethylrhodamine methyl ester. Importantly, cyclosporin A and other MPT blockers prevent increased permeability of mitochondria to calcein and loss of the mitochondrial membrane potential (Fig. 1). Furthermore, the MPT blockers prevent onset of cell death, which strongly supports the hypothesis that the MPT is a causative mechanism in these models of acute necrotic cell killing.

THE pH PARADOX AND OXIDATIVE INJURY

During ischemia, tissue pH decreases rapidly. Rather than aggravating injury, this acidosis delays the onset of cell death. However, restoration of normal pH after reperfusion accelerates cell killing, a phenomenon called the pH paradox (7). ROS do not cause pH-dependent cell killing, since anaerobic reperfusion at normal pH causes as much cell killing as reperfusion in the presence of oxygen. Although many factors may contribute to pH-dependent reperfusion injury, onset of the MPT is perhaps the most important one. When anoxic hepatocytes at pH 6.5 are “reperfused” with normoxic buffer at pH 7.4, the MPT visualized by calcein redistribution occurs as intracellular pH rises to ~7 (34). Subsequently, the cells lose viability. If, at the time of reperfusion, the cells are exposed to cyclosporin A, then calcein does not redistribute from the cytosol into the mitochondria. Instead, the mitochondria repolarize, and cell viability is retained. Similarly, reoxygenation with acidic buffer blocks onset of the MPT, permitting...
mitochondrial repolarization and retention of viability. Thus, the MPT is the major mechanism promoting onset of acute necrotic cell killing in this model of ischemia-reperfusion injury.

The MPT also plays a causative role in cell killing caused by tert-butyl hydroperoxide (t-BuOOH), the short chain analog of lipid hydroperoxides. In cultured hepatocytes, onset of the MPT precedes t-BuOOH-induced cell killing, and both the MPT and cell death are prevented by the MPT blocker, trifluoperazine (31). Oxidation of mitochondrial pyridine nucleotides (NADH and NADPH), an increase of mitochondrial matrix free Ca\(^{2+}\), and generation of mitochondrial ROS all precede development of the MPT (27, 28). Just as in isolated mitochondria, each of these mitochondrial changes helps to promote onset of the MPT and subsequent cell death. Measures that delay or prevent pyridine nucleotide oxidation, the increase of mitochondrial Ca\(^{2+}\), and mitochondrial ROS formation also delay or prevent the MPT and subsequent cell death. Mitochondrial ROS are also formed during excitotoxic injury to neurons, and several recent reports implicate the MPT as a causative mechanism in excitotoxicity (29, 36, 38).

**APOPTOSIS**

Necrosis and apoptosis have long been viewed as fundamentally different processes. Necrotic cell death results from acute metabolic disruption with ATP depletion, ion disregulation, mitochondrial and cellular swelling, and activation of degradative enzymes. These processes culminate in rupture of the plasma membrane and loss of intracellular proteins, metabolites, and ions. In contrast, apoptosis represents a special form of cellular differentiation that leads to the orderly resorption of target cells without severe impairment of cellular metabolism. Specific signals, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and Fas ligand, trigger onset of apoptosis through activation of a cascade of cysteine-aspartate proteases called caspases (reviewed in Ref. 35).

Despite the differences between necrotic and apoptotic cell death, the MPT also plays a causative role in apoptosis as well. In a cell free system combining purified nuclei and mitochondria, onset of the MPT induces release of soluble mitochondrial factors that activate caspases and initiate apoptotic nuclear changes (17). These factors include an apoptosis-inducing factor (AIF) and cytochrome \(c\), the latter a diffusible electron carrier in the intermembrane space between the mitochondrial inner and outer membrane (22). Breakage of the outer membrane after MPT-induced mitochondrial swelling is one likely mechanism by which cytochrome \(c\) and AIF are released.

**MECHANISM OF CYTOCHROME C RELEASE**

Cytochrome \(c\) is the best studied of the proapoptotic factors released by mitochondria. In the cytosol, cytochrome \(c\) binds to apoptosis-activating factor-1 (20). Additional binding of ATP (or dATP) then activates caspase 9, which in turn activates caspase 3. Finally, caspase 3 stimulates the so-called executioner pathway of apoptosis, leading to poly(ADP-ribose)polymerase (PARP) cleavage, internucleosomal DNA hydrolysis, cell shrinkage, chromatin margination, and nuclear loblution. Caspases are also involved upstream of release of cytochrome \(c\) from mitochondria. Binding of TNF-\(\alpha\) and Fas ligand to their receptors activates caspase 8. Recent evidence indicates that caspase 8 cleaves Bid, a member of the Bcl2 family of proteins, which then translocates to mitochondria to induce cytochrome \(c\) release (19, 25) (see Fig. 2).

Whether the MPT actually occurs in cellular apoptosis remains controversial, and some studies claim that cytochrome \(c\) release during apoptosis occurs without mitochondrial depolarization (16, 39). However, during apoptosis in hepatocytes induced by TNF-\(\alpha\), onset of the MPT as directly visualized by confocal microscopy (Fig. 3) precedes cytochrome \(c\) release, activation of caspase 3, PARP cleavage, internucleosomal DNA deg...

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**Fig. 2.** Scheme of molecular events in tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\))-induced apoptosis. TNF-\(\alpha\) binding to its receptor (TNFR) activates caspase 8 via the adapter proteins, TRADD and FADD. Bid is cleaved and translocated to the mitochondria. Onset of the MPT leads to cytochrome \(c\) release and its binding to apoptosis-activating factor-1 (APAF-1) and ATP (not shown), which is followed by a cascade of caspase 9 and caspase 3 activation, resulting in apoptotic cell death. Signaling through another adapter protein, Traf, activates the nuclear transcription factor, NF-\(\kappa\)B, which leads to anti-apoptotic gene expression acting upstream of mitochondria. Expression of an I\(\kappa\)B super-repressor, I\(\kappa\)B-AA, inhibits the activation of NF-\(\kappa\)B and is permissive for TNF-\(\alpha\)-induced apoptosis. Expression of crmA inhibits the upstream caspase 8 and blocks the MPT after TNF-\(\alpha\) addition, whereas inhibition of downstream caspase 3 with DEVD-cho prevents apoptosis but not the onset of the MPT. Expression of aFADD, a truncated FADD, also blocks apoptotic signaling upstream of the MPT.
radiation, and the morphological changes of apoptosis (4). Cyclosporin A prevents the MPT induced by TNF-α and blocks cytochrome c release, caspase 3 activation, and apoptosis. As hepatocytes undergo apoptosis in this model, onset of the MPT occurs progressively through the mitochondria of each cell, and 4 or more hours pass between onset of the MPT in the first and last mitochondrion. For this period of time, polarized mitochondria coexist with depolarized mitochondria that have undergone MPT, which is consistent with reports of cytochrome c release from cells still containing polarized mitochondria. Another recent study evaluated the release from mitochondria of a transfected fusion protein of cytochrome c and green fluorescent protein (GFP) during staurosporin-induced apoptosis to PC6 pheochromocytoma cells (13). Cytochrome c-GFP release accompanied but did not precede mitochondria depolarization, consistent with the hypothesis that mitochondrial swelling and outer membrane rupture after the MPT cause cytochrome c release. Thus the MPT is in the middle of the apoptotic signaling cascade, downstream of receptor binding, caspase 8 activation, and Bid cleavage and upstream of cytochrome c release and the activation of caspases 3 and 9 (Fig. 2).

ROLE OF ATP IN DIRECTING APOPTOTIC AND NECROTIC CELL KILLING

If the MPT causes both necrosis and apoptosis, what factor determines how a cell will die? Apoptosis requires ATP, both in cells and cell-free systems (8, 18, 22), whereas intracellular ATP actually prevents onset of necrotic cell death (30). Thus the effect of the MPT on ATP may determine whether necrotic cell death or apoptosis ensues (Fig. 4). In hepatocytes, rapid onset of the MPT after Ca²⁺ ionophore treatment leads to profound ATP depletion and necrotic cell death within 45 min. However, if ATP levels are maintained by a glycolytic substrate such as fructose in the presence of oligomycin to block ATP hydrolysis by the uncoupler-stimulated mitochondrial ATPase, this necrotic killing is prevented. Nonetheless, the MPT still occurs, and apoptosis develops several hours later, which is nearly completely inhibited by cyclosporin A (33). Thus the MPT is mediating both necrosis and apoptosis. When the MPT depletes ATP, necrotic cell death results, but, when the MPT occurs without severe ATP depletion, apoptosis develops instead (Fig. 4). Presumably, if ATP depletion develops during the progression of apoptosis, necrotic cell death will intervene to produce the secondary necrosis that is so often associated with apoptosis.

NECRAPOPTOSIS

The MPT is a pathophysiological mechanism shared by both apoptosis and necrosis. As a consequence, the long-held distinction between apoptotic and necrotic cell death becomes blurred. Indeed, in tissue injury due to ischemia-reperfusion, toxic chemicals, and viral infection, apoptotic and necrotic features often coexist. This has led to controversies as to whether the apparent apoptosis of acute chemical toxicity and reperfusion injury is really necrosis in disguise (9, 32). Presumably, one might also argue that the apparent necrosis in acute viral disease is actually apoptosis.

Controversies among scientists are generally resolved in one of two ways: 1) nobody is right and 2) everyone is right. In regard to the issue of whether apoptosis or necrosis is the predominant mode of cell death in chemical toxicity and ischemia-reperfusion injury, the facts support the second resolution. Features characteristic of necrotic and apoptotic cell death
are not only occurring in the same tissues but simultaneously in the same cells. Unfortunately, implicit in our nomenclature is the assumption that either one or the other form of cell killing must occur. Hence, a new term such as “necrapoptosis” is needed. By necrapoptosis, I mean a process that begins with a common death signal or toxic stress but that culminates in either cell lysis (necrotic cell death) or programmed cellular resorption (apoptosis), depending on other modifying factors. Cell death mediated by the MPT illustrates this idea. When onset of the MPT is rapid and cellular ATP levels drop dramatically, then early cell lysis ensues. If progression of the MPT is slower, or if other sources of ATP generation are available, then profound ATP depletion is avoided, allowing apoptotic signaling to proceed. Later if ATP levels finally collapse, cell lysis supervenes in a pattern of secondary necrosis (Fig. 4). Pure apoptosis and pure necrosis represent extremes in the spectrum of necrapoptotic responses, but the more typical response of tissues and cells to injurious stresses and other death signals is a mixture of events associated with apoptotic and necrotic cell death.

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