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Forum Review

Role of Mitochondrial Inner Membrane Permeabilization in Necrotic Cell Death, Apoptosis, and Autophagy

JOHN J. LEMASTERS,[1] TING QIAN,[1] LIHUA HE,[1] JAE-SUNG KIM,[1]
STEVEN P. ELMORE,[1] WAYNE E. CASCIO,[2] and DAVID A. BRENNER[2]

ABSTRACT

Inhibition of mitochondrial oxidative phosphorylation progresses to uncoupling when opening of cyclosporin A-sensitive permeability transition pores increases permeability of the mitochondrial inner membrane to small solutes. Involvement of the mitochondrial permeability transition (MPT) in necrotic and apoptotic cell death is implicated by demonstrations of protection by cyclosporin A against oxidative stress, ischemia/reperfusion, tumor necrosis factor-α exposure, Fas ligation, calcium overload, and a variety of toxic chemicals. Confocal microscopy directly visualizes the MPT in single mitochondria within living cells from the translocation of impermeant fluorophores, such as calcein, across the inner membrane. Simultaneously, mitochondria release potential-indicating fluorophores. Subsequently, mitochondria swell, causing outer membrane rupture and release of cytochrome *c* and other proapoptotic proteins from the intermembrane space. *In situ* a sequence of decreased NAD(P)H, increased free calcium, and increased reactive oxygen species formation within mitochondria promotes the MPT and subsequent cell death. Necrotic and apoptotic cell death after the MPT depends, in part, on ATP levels. If ATP levels fall profoundly, glycine-sensitive plasma membrane permeabilization and rupture ensue. If ATP levels are partially maintained, apoptosis follows the MPT. The MPT also signals mitochondrial autophagy, a process that may be important in removing damaged mitochondria. Cellular features of necrosis, apoptosis, and autophagy frequently occur together after death signals and toxic stresses. A new term, *necrapoptosis*, describes such death processes that begin with a common stress or death signal, progress by shared pathways, but culminate in either cell lysis (necrosis) or programmed cellular resorption (apoptosis), depending on modifying factors such as ATP. *Antioxid. Redox Signal.* 4: 769–781.

INTRODUCTION

ISCHEMIA is a condition of impaired blood supply to an organ or tissue in which oxygen delivery is inadequate to meet tissue oxygen demand. As tissues consume oxygen, hypoxia progresses to nearly absolute anoxia (pO_2 << 1 Torr) because of the high affinity of mitochondrial cytochrome oxidase for oxygen. In hepatocytes and several other cell types, formation of plasma membrane protrusions called blebs is an early structural change in hypoxia, reflecting disruption of volume regulation and cytoskeletal organization after ATP depletion (54, 55). Blebs contain cytosol and endoplasmic reticulum, but usually exclude mitochondria, lysosomes, and other large organelles. As blebs form, cisternae of endoplasmic reticulum dilate, mitochondria round up and swell moderately, and cell volume increases by 30–50%.

These early changes reflect reversible hypoxic injury when full recovery of cells after reoxygenation is still possible. Late in hypoxic stress, however, a distinct metastable state develops that is characterized by mitochondrial permeabilization, lysosomal disruption, bleb coalescence and growth, leakage of low-molecular-weight anionic solutes across the plasma membrane, and accelerated cell swelling (56, 66, 100). This metastable state then culminates in bleb rupture

Departments of [1]Cell and Developmental Biology and [2]Medicine, University of North Carolina, Chapel Hill, North Carolina. Dr. Elmore's present address is Vrije Universiteit, Laboratory for Physiology, Amsterdam, The Netherlands.

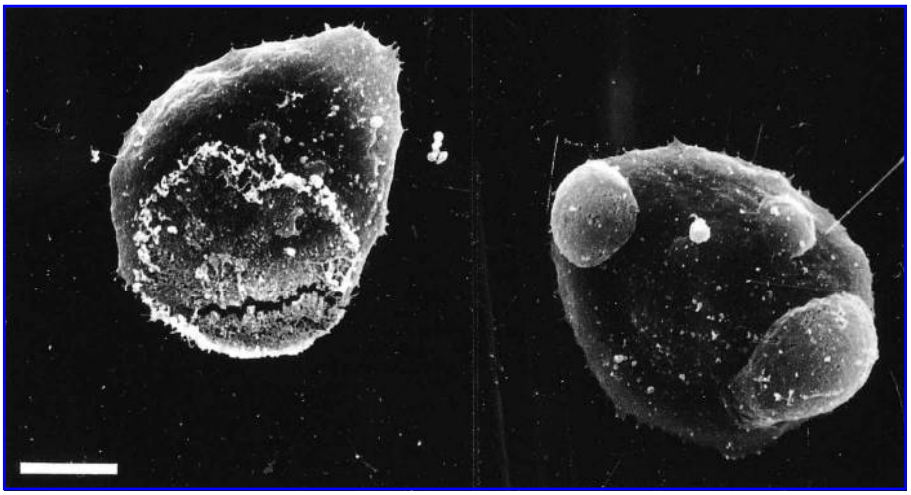

FIG. 1. Scanning electron micrograph of plasma membrane rupture at onset of necrotic cell death. Cultured rat hepatocytes were exposed to cyanide and iodoacetate, inhibitors of respiration and glycolysis, respectively. The plasma membrane of the hepatocyte on the right has burst in association with onset of necrotic cell death. The hepatocyte on the left is blebbed, but the plasma membrane is intact and the cell has not yet lost viability. Bar = 5 μm. Adapted from (66).

and loss of the plasma membrane permeability barrier (Fig. 1). Such bleb rupture brings about irreversible injury and onset of necrotic cell death. The abrupt failure of the plasma membrane permeability barrier also causes release of cytosolic contents, including enzymes and metabolites, uptake of extracellular death markers like trypan blue, and collapse of all plasmalemmal electrical and ionic gradients (56, 66, 100). These consequences of bleb rupture make continued life of the cell no longer possible.

GLYCINE CYTOPROTECTION

The amino acid glycine has the remarkable property of protecting against hypoxic cell killing in a variety of cell types (21, 61, 72, 94). Glycine does not prevent ATP depletion, but rather blocks progression into the metastable state. In ATP-depleted Madin–Darby canine kidney (MDCK) cells, glycine blocks development of porous plasma membrane defects that enlarge from passing 4,000-Da solutes to passing large dextrans of 70,000 Da or more (23). In hepatic sinusoidal endothelial cells, glycine blocks opening of organic anion channels, which are permeable to chloride and polyvalent organic anions up to a molecular mass of at least 600 Da, but which are not permeable to similarly sized organic cations or larger molecular weight dextrans (71). Opening of these organic anion channels leads to the accelerated cellular swelling of the metastable state (Fig. 2). Rapid swelling is the consequence of simultaneous Cl^- entry through glycine-sensitive anion channels and Na^+ entry through monovalent cation channels. The latter cation channels open early in hypoxia (16, 35, 42), whereas anion channel opening is a penultimate event that initiates the metastable state and cell death. Rapid swelling during the metastable state is driven by the oncotic (colloid osmotic) pressure of macromolecules in the cytosol. Continued swelling leads to membrane stretching and ultimately bursting of the plasma membrane. Such stretching may account for the formation and growth of nonspecific porous defects in the plasma membrane just preceding cell death, as observed in MDCK cells. Once the plasma membrane bursts, swelling ceases because release of intracellular macromolecules eliminates the oncotic pressure gradi-

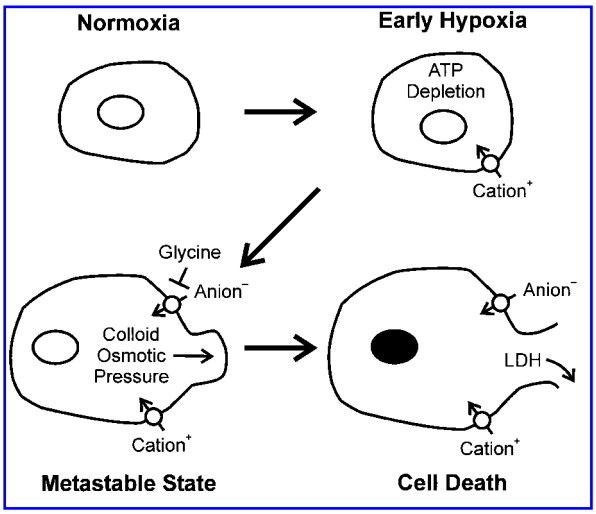

FIG. 2. Glycine-sensitive plasma membrane permeabilization leading to hypoxic cell death. Cation gradients (Na^+ and K^+) collapse early during hypoxia due to inhibition of the Na,K-ATPase and opening of monovalent cation channels. However, little cell swelling occurs because the plasma membrane remains impermeable to anions. Later, following ATP depletion, glycine-sensitive anion channels open, which initiates anion entry, accelerated bleb formation, and onset of the metastable state. As the plasma membrane stretches, defects in the lipid bilayer develop, leading to membrane rupture. Dyes like trypan blue and propidium now enter to label the nucleus, and cytosolic enzymes are released. With abrupt and complete loss of the plasma membrane permeability barrier, the cell is dead. LDH, lactate dehydrogenase. Adapted from (71).

ent driving the volume expansion. Since permeabilization of first mitochondria and then lysosomes occurs at onset of the metastable state, a hydrolytic enzyme, such as a protease, or other factor released by these organelles may be important to open the glycine-gated anion channel (100).

ATP DEPLETION AS A KEY EVENT LEADING TO NECROTIC CELL DEATH AFTER ISCHEMIA/HYPOXIA

Cessation of mitochondrial ATP formation by oxidative phosphorylation is the fundamental stress of anoxic and ischemic injury. Rescue of cells from hypoxic killing by glycolytic substrates demonstrates directly the importance of ATP depletion in hypoxic injury (Fig. 3) (1, 67, 72). Glycolysis only partially replaces lost mitochondrial ATP production, and 15–20% of normal ATP levels is sufficient to prevent onset of anoxic cell killing. Glycolytic substrates, such as glucose, fructose, and endogenous glycogen, prevent anoxic killing almost entirely, but protection depends on the metabolic capabilities of specific cell types. For example, glucose does not protect hepatocytes against anoxic injury, because hepatocytes lack hexokinase, the enzyme that catalyzes the first reaction of glycolysis in most cells. Instead, hepatocytes possess glucokinase, a relatively sluggish enzyme with a high K_m for glucose and a relatively low V_{max}. Glucokinase is metabolically appropriate for hepatocytes, because the liver has the important function of stabilizing blood glucose to ~5 m*M*. Even during anoxia, glucose consumption by liver is low, because rapid hepatic glucose utilization would otherwise cause systemic hypoglycemia. In hepatocytes, fructose protects hepatocytes against anoxic injury, because hepatocytes contain fructokinase, an enzyme with a low K_m and high V_{max} that feeds fructose into the glycolytic pathway.

Glycogen also supports anaerobic glycolysis, and glycogen-rich hepatocytes from fed rats are much more resistant to anoxic killing than glycogen-depleted hepatocytes from fasted rats (8). Glycolytic substrate also protects against hepatocellular killing by several toxic chemicals (67, 96). This observation implies that mitochondrial ATP production is a principal target of toxic cell killing. Similarly in humans, glycogen depletion after fasting predisposes to acetaminophen-induced liver damage (95). This effect is usually attributed to a moderate decline of hepatic glutathione during fasting, but may simply be the consequence of the inability of glycolysis to substitute for an interruption of mitochondrial ATP production.

Fructose metabolism via the fructokinase reaction causes ATP and P_i to decrease, an effect sometimes presumed to represent fructose toxicity. Glucose causes a similar decline of ATP and P_i in hexokinase-containing cells. Because decreased P_i offsets decreased ATP, fructose-treated livers maintain their ATP/ADP·P_i ratios, which means that the free energy available from ATP hydrolysis does not drop. Furthermore, during anoxia and respiratory inhibition when ATP declines profoundly, fructose increases ATP substantially, and this ATP generation prevents cell killing (68).

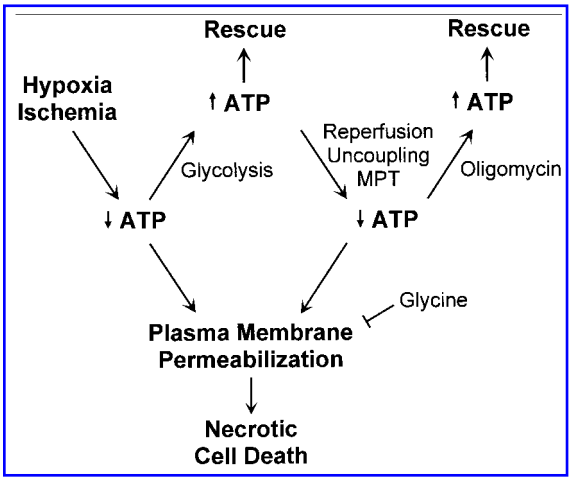

FIG. 3. Mitochondrial injury during ischemia/reperfusion. Anoxia during ischemia inhibits oxidative phosphorylation, which leads to ATP depletion, plasma membrane permeabilization, and necrotic cell death. Glycine blocks plasma membrane permeabilization and onset of cell death downstream of ATP depletion. Glycolysis restores ATP and prevents loss of cell viability. Reperfusion induces the mitochondrial permeability transition (MPT) and mitochondrial uncoupling, which activates the mitochondrial ATPase. The mitochondrial ATPase futilely hydrolyzes ATP made available by glycolysis, which overcomes the protective effect of glycolysis. By inhibiting the mitochondrial ATPase, oligomycin prevents ATP depletion and rescues cells from necrotic cell death provided glycolytic substrate is present. Adapted from (68).

MITOCHONDRIAL UNCOUPLING IN CELL INJURY

Uncoupling represents a more severe form of metabolic disruption to mitochondria than simple inhibition of mitochondrial respiration or ATP synthesis. Uncoupling causes collapse of pH and electrical gradients (ΔpH and $\Delta\Psi$, respectively) across the mitochondrial inner membrane and activates the mitochondrial F_1F_0 ATPase (ATP synthase) (Fig. 3). Because of mitochondrial ATPase activation, glycolytic ATP becomes futilely hydrolyzed, and glycolytic substrates can no longer rescue cells from acute necrotic cell death. Under conditions of uncoupling, oligomycin, a specific inhibitor of the mitochondrial F_1F_0 ATPase, prevents mitochondrial hydrolysis of glycolytic ATP after uncoupling and restores cytoprotection (67, 68). Thus, mitochondrial injury can progress from simple inhibition of respiration and oxidative phosphorylation, which glycolysis can protect against, to uncoupling and active hydrolysis of ATP, which glycolysis cannot overcome (Fig. 3).

Many toxicants induce cytotoxicity that glycolytic substrates protect against, whereas for others protection only occurs if glycolytic ATP formation is combined with oligomycin. Such a requirement for oligomycin implicates mitochondrial uncoupling as the mechanism of toxicity, as occurs with the calcium ionophore Br-A23187, often used as a model of Ca^{2+}-dependent cytotoxicity, the monovalent cation

ionophore gramicidin D, and the oxidant chemical, *tert*-butyl hydroperoxide (*t*-BuOOH) (40, 67).

CYTOPROTECTION BY ACIDOTIC pH AGAINST HYPOXIC AND TOXIC CELL KILLING

Ischemia initiates anaerobic glycolysis and the hydrolysis of high-energy nucleoside phosphates (ATP, GTP, etc.). Both events promote the accumulation of hydrogen ions, and cell and tissue pH in ischemia can rapidly decrease by a unit or more (29, 97). This naturally occurring acidosis in ischemia actually serves to delay the onset of necrotic cell death in a variety of cell types, even though acidosis does little or nothing to prevent profound depletion of cellular ATP and other high-energy intermediates (7, 28, 29). The mechanism by which acidosis protects against injury remains incompletely understood. Acidotic pH suppresses degradative enzymes activated by hypoxic stress, such as phospholipases and proteases (2). Acidotic pH also suppresses onset of the mitochondrial permeability transition (MPT) (see below).

THE pH PARADOX IN ISCHEMIA/REPERFUSION INJURY

Reperfusion after ischemia paradoxically worsens cell injury and causes tissue necrosis within minutes. Much of this cell killing is linked to the recovery of pH from acidotic to normal after reperfusion, because reoxygenation at low pH can prevent cell killing almost entirely, whereas recovery of normal pH without reoxygenation causes nearly the same cell killing as pH recovery with reoxygenation. This pH-dependent reperfusion injury is termed the pH paradox (6, 20, 28, 37, 99).

Changes of intracellular pH mediate the protection conferred by acidosis and the exacerbation of injury of the pH paradox. Measures that increase intracellular acidosis increase protection, whereas measures that promote intracellular alkalinization increase cell killing. For example, when an ionophore like monensin accelerates recovery of intracellular pH during reperfusion, cell killing occurs more quickly. By contrast, suppression of the recovery of intracellular pH during reperfusion by Na^+/H^+ exchange blockade with dimethylamiloride or Na^+-free medium prevents reperfusion-induced necrotic cell killing almost completely. pH-dependent cell killing is directly linked to changes of intracellular pH and occurs independently of pH-dependent secondary changes of cytosolic Na^+ and Ca^{2+} (6, 29, 37, 44, 80).

MITOCHONDRIAL PERMEABILITY TRANSITION

The mitochondrial permeability transition (MPT) occurs by opening of a pore, the permeability transition (PT) pore, which conducts freely solutes of molecular weight less than ~1,500 Da (4). As a consequence, mitochondria depolarize, uncouple, and undergo large amplitude swelling. Ca^{2+}, P_i, reactive oxygen and nitrogen species, and numerous reactive chemicals induce PT pore opening, whereas Mg^{2+}, low pH, and the immunosuppressant drug, cyclosporin A (CsA), block pore conductance. Pore blockade by CsA is unrelated to its immunosuppressive action, because several nonimmunosuppressive CsA derivatives also block the pore. Moreover, other immunosuppressants, such as tacrolimus, have no effect. Patch clamping shows that individual pores have very high conductance, and opening of just one PT pore may be sufficient to induce mitochondrial depolarization (91).

There is much speculation that the PT pore is comprised of a complex of proteins, including the adenine nucleotide translocator (ANT) protein in the mitochondrial inner membrane, the voltage-dependent anion channel (also called porin) in the outer membrane, the CsA-binding protein cyclophilin D (CypD) in the mitochondrial matrix, and possibly other proteins, such as the proapoptotic protein, Bax (10, 19, 76). This complex of proteins implies that PT pores span the inner and outer membranes, presumably at Hackenbrock's contact sites between the two membranes (34). This model is consistent with a variety of experimental observations, especially the inhibition and activation of the MPT by bongkrekic acid and atractyloside, ligands of ANT, and MPT blockade by CsA, which binds to CypD (18, 36).

Other experimental findings are more difficult to explain by this model of PT pore structure. Although Ca^{2+}-dependent activation and CsA inhibition of the MPT are considered hallmarks of the MPT, the MPT can also occur in the presence of CsA and the absence of Ca^{2+} (12, 27). Recent evidence indicates that the PT pores have two functional modes: a regulated mode that is Ca^{2+}-dependent and inhibited by CsA and Mg^{2+} and an unregulated mode that does not require Ca^{2+} for pore opening and is not inhibited by CsA or Mg^{2+} (39). In general, low-level MPT induction opens regulated pores, whereas stronger induction opens unregulated pores. Moreover, although PT pore-like conductance can be reconstituted with ANT alone or in combination with other proteins (13, 19, 62), other purified mitochondrial anion carriers, such as the aspartate/glutamate and phosphate carriers, can be converted to unspecific pores (22, 85). Additionally, small exogenous amphipathic peptides (*e.g.*, alamethicin and mastoparan) and the mitochondrial matrix targeting sequence of cytochrome *c* oxidase subunit IV induce an MPT that is Ca^{2+}-dependent and inhibited by CsA (50, 78).

Although PT pores are often assumed to be a normal constituent of mitochondrial membranes, induction of the MPT typically requires mitochondrial membrane damage, such as attack by oxygen radicals and protein disulfide cross-linking agents (92). To explain how chemical attack on membrane proteins causes opening of first regulated PT pores and then unregulated PT pores, a new model of PT pore formation and regulation has been recently proposed (Fig. 4) (39). This model proposes that pores form by aggregation of damaged integral membrane proteins attacked by oxidant and other chemicals. Damage causes misfolding and exposure of hydrophilic residues to the bilayer phase of the membrane. As a consequence, protein clustering occurs at these hydrophilic surfaces to enclose aqueous channels that conduct low-molec-

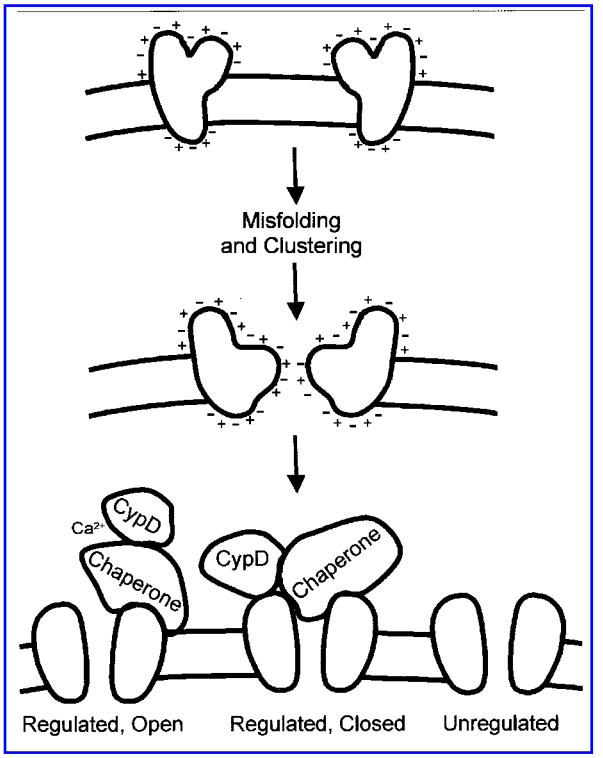

FIG. 4. New model of PT pore structure and regulation. Misfolding of native membrane proteins after oxidative damage and other perturbations exposes hydrophilic residues to the bilayer phase, which cluster and enclose aqueous channels conducting low-molecular-weight solutes. Chaperone-like proteins and cyclophilin D (CypD) bind to amphipathic protein clusters to block pore conductance. Increased Ca^{2+} perturbs the protein cluster/chaperone complexes to open an open conductance state that is inhibited by CsA. Unregulated pores form when protein clusters exceed chaperones available to block and regulate their conductance. Adapted from (39).

ular-weight solutes. ANT molecules are often involved in this clustering for the simple reason that on a molar basis ANT is the most abundant integral membrane protein in the mitochondrial inner membrane. However, conductance through these misfolded protein clusters is initially blocked by chaperone-like proteins. Chaperone and CypD binding to misfolded protein clusters creates regulated PT pores and allows Ca^{2+} to induce CsA-sensitive pore opening. When protein clusters exceed chaperones available to block conductance, then unregulated pore opening occurs. This new model, although speculative, attempts to explain many of the properties of PT pores that older models cannot account for.

THE MITOCHONDRIAL PERMEABILITY TRANSITION IN PH-DEPENDENT REPERFUSION INJURY

pH below 7.0 inhibits onset of the MPT, and CsA is remarkably protective against ischemia/reperfusion injury in several systems, even when added only during the reperfusion phase (31, 63, 80). Laser scanning confocal microscopy confirms directly onset of the MPT in pH-dependent reperfusion injury. When hepatocytes subjected to anoxia at acidotic pH are reoxygenated at normal pH, mitochondria initially begin to repolarize (Fig. 5). Then, after several minutes, the nonspecific permeability of the mitochondrial inner membrane to cytosolic fluorophores increases abruptly as intracellular pH rises to ~7.0, and simultaneously mitochondrial $\Delta\Psi$ collapses. Cell death then occurs soon after. After reoxygenation at acidic pH or at normal pH with CsA, mitochondrial permeabilization, depolarization, and cell death do not occur. Instead, blebs disappear and volume regulation recovers, indicating restoration of ATP-dependent activities (Fig. 5). Thus, the MPT represents an important causative mechanism in the pathogenesis of pH-dependent reperfusion injury (80).

MITOCHONDRIAL INJURY FROM REACTIVE OXYGEN AND NITROGEN SPECIES

Reoxygenation of ischemic and hypoxic tissues promotes the formation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$). Sources of ROS include xanthine oxidase utilizing xanthine and hypoxanthine generated after ATP degradation and NADPH oxidase in macrophages, neutrophils, and other cells. In addition, the respiratory chain of metabolically disrupted mitochondria are a major source of ROS. Transition metal ions, such as free iron and copper, catalyze formation of highly reactive and toxic hydroxyl radicals ($OH\cdot$) from H_2O_2 and $O_2^{\cdot -}$ by the Fenton reaction. Iron also catalyzes lipid peroxidation chain reactions sustained by lipid alkyl and peroxyl radicals. Desferal, an iron chelator, blocks these reactions. In a diffusion-limited nonenzymatic reaction, $O_2^{\cdot -}$ also combines with nitric oxide ($NO\cdot$) to form peroxynitrite ($OONO^-$), an important toxic intermediate in oxidative tissue injury, which decomposes to a hydroxyl radical-like species (33).

Oxidative stress, ROS, and peroxynitrite all promote onset of the MPT in isolated mitochondria. ROS also cause the MPT in intact cells, as shown in hepatocytes treated with tert-butyl hydroperoxide (*t*-BuOOH), a short chain analogue of the lipid hydroperoxides formed during oxidative stress and ischemia/reperfusion (69). In hepatocytes, *t*-BuOOH initiates a chain of events that culminates in cell lysis and death. Initially, *t*-BuOOH causes oxidation of mitochondrial pyridine nucleotides (NADH and NADPH) and glutathione within several seconds. This oxidation is followed by an increase of intramitochondrial free Ca^{2+}, which stimulates mitochondrial ROS formation. Interestingly, the increase of intramitochondrial Ca^{2+} precedes changes of cytosolic Ca^{2+}. ROS formation then initiates PT pore opening, mitochondrial depolarization, ATP depletion, and cell death (15, 70). A similar sequence may contribute to pH-dependent reperfusion injury, because intramitochondrial free Ca^{2+} increases early after reperfusion and is then followed by mitochondrial ROS formation, inner membrane permeabilization, and cell death (45).

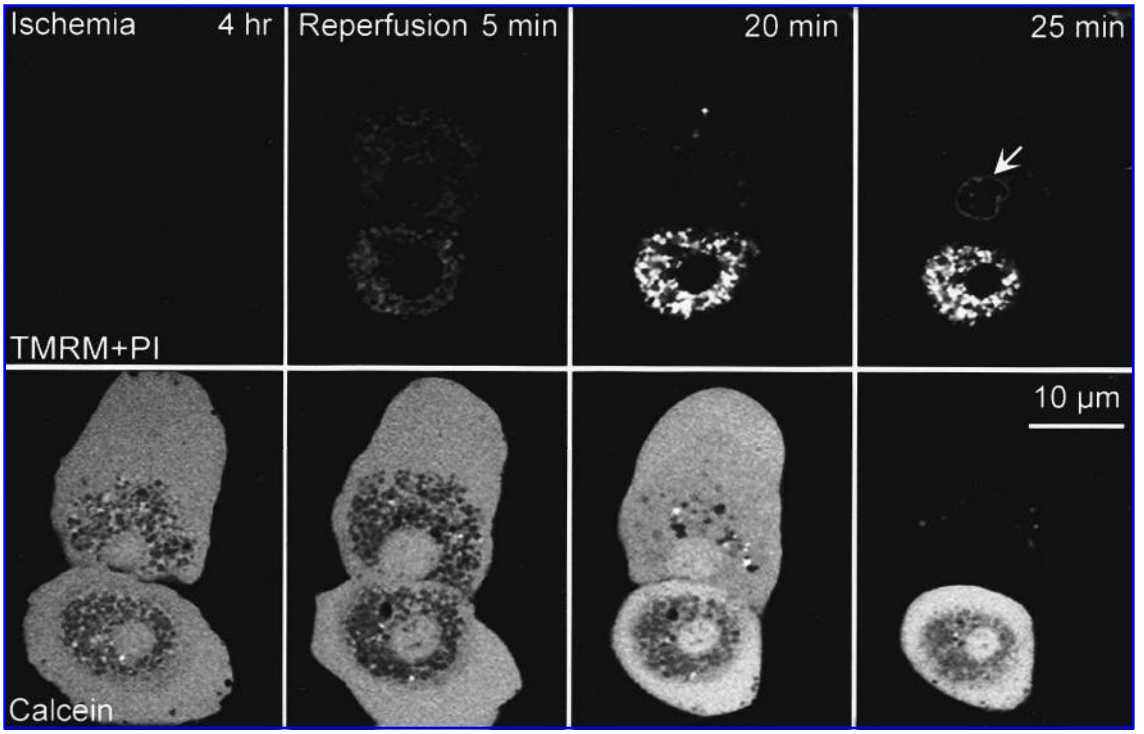

FIG. 5. Onset of the MPT after simulated ischemia and reperfusion to hepatocytes. Rat hepatocytes were loaded with green-fluorescing calcein (**lower panels**) and red-fluorescing tetramethylrhodamine methyl ester (TMRM, **upper panels**) and subjected to anoxia at pH 6.2 to simulate ischemia. At the end of 4 h, mitochondria were small dark round voids in confocal images of calcein fluorescence, which indicated that the mitochondrial inner membrane was still impermeable to this 623-Da fluorophore in the cytosol. Simultaneously, mitochondria were depolarized and did not accumulate TMRM, a membrane potential-indicating fluorophore. After reoxygenation at pH 7.4 to simulate reperfusion, mitochondria began to take up TMRM within 5 min. After 20 min, mitochondria of one of the hepatocytes in the field then lost TMRM labeling and filled with calcein, indicating mitochondrial depolarization and onset of the MPT, respectively. This hepatocyte then lost viability after 25 min, as indicated by loss of calcein and nuclear uptake of propidium iodide (PI; arrow). The other hepatocyte in the field retained viability, and its mitochondria continued to accumulate TMRM and exclude calcein after 25 min of reperfusion. Adapted from (80).

APOPTOSIS IN OXIDATIVE AND REPERFUSION INJURY

To this point, acute cytotoxicity characterized by cell swelling and plasma membrane failure has been discussed. These events constitute necrotic cell death, the process of which is called oncosis (60). Another mode of cell death is apoptosis in which cell deletion occurs without the inflammation, scarring, and release of cellular contents that characterize necrotic cell death. Unlike necrotic cell death, apoptosis requires ATP. Typical features of apoptosis include cell shrinkage, activation of cysteine-aspartate proteases called caspases, and internucleosomal DNA degradation. A wide range of physiological and nonphysiological signals initiate apoptosis in various systems.

As with necrotic cell death, mitochondria play a major role in apoptosis. In many forms of apoptosis, mitochondria release proteins from the intermembrane space between the inner and outer membrane that propagate apoptotic signaling. These proteins include the loosely bound respiratory protein cytochrome *c*, apoptosis-inducing factor (AIF), and Diablo-SMAC (24, 59, 89, 90). Together with ATP (or dATP) and pro-caspase 9, cytochrome *c* forms a complex with apoptosis-inducing factor-1 (APAF-1) to yield a proteolytically activated caspase 9 (Fig. 6) (58). Activated caspase 9 then proteolytically activates pro-caspase 3 to caspase 3 to initiate the final execution stages of apoptosis, including cell shrinkage, zeiotic surface blebbing, internucleosomal DNA hydrolysis, chromatin margination, and nuclear lobulation. Diablo-SMAC released from mitochondria binds and neutralizes caspase inhibitors to allow this activation cascade to progress, whereas AIF translocates to the nucleus to promote non–caspase-dependent chromatin degradation.

Other caspases, such as caspase 8, act upstream of mitochondria in this apoptotic signaling pathway (Fig. 6). For example, in tumor necrosis factor-α (TNFα) and Fas-dependent apoptosis, ligand–receptor interaction produces receptor trimerization and binding of various adapter proteins, such as the TNFα receptor-associateddeath domain protein (TRADD) and the Fas-associated death domain protein (FADD). These complexes, sometimes called death-inducing signaling complexes (DISC), recruit and activate pro-caspase 8. Caspase 8 then cleaves Bid, a member of the proto-oncogene Bcl-2 family of

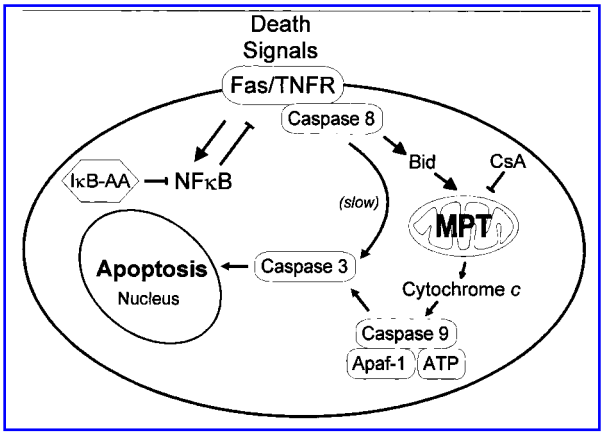

FIG. 6. Molecular signaling in death receptor-induced apoptosis. Binding of death signals (*e.g.*, Fas ligand, TNFα) to their corresponding death receptors [Fas or TNRα receptor (TNFR)] activates caspase 8. Caspase 8 cleaves Bid, which then translocates to mitochondria. Subsequently, the MPT occurs, leading to release of cytochrome *c*. CsA blocks this mitochondrial permeabilization. Cytochrome *c* released by mitochondria binds to apoptosis-inducing factor 1 (Apaf-1). In the presence of ATP, this complex activates caspase 9, which in turn proteolytically activates caspase 3 to induce the major biochemical and morphological manifestations of apoptosis. Death receptor signaling also leads to activation of the nuclear transcription factor, NFκB, which induces antiapoptotic gene expression acting upstream of mitochondria. Adenoviral expression of an IκB super-repressor, IκB-AA, inhibits NFκB activation and promotes TNFα-induced apoptosis in hepatocytes. Caspase 8 also directly activates caspase 3 bypassing mitochondria, but this occurs slowly in hepatocytes.

proteins, to a 15-kDa truncated form (tBid). tBid then translocates to mitochondria and induces release of cytochrome *c* and other proteins from the intermembrane space. Other proapoptotic members of the Bcl2 family, such as Bax, Bak, and Bad, can also promote outer membrane permeabilization. Still other family members, such as Bcl2 and Bcl_{XL}, block cytochrome *c* release and prevent apoptotic signaling through mitochondria (49).

The mechanisms by which cytochrome *c* and other intermembrane proteins are released during apoptosis are the subject of ongoing controversy. Recent work indicates that cytochrome *c* release induced by tBid requires one other proapoptotic Bcl-2 family member, either Bax or Bak (93). Double knockout fibroblasts lacking both Bax and Bak, but not single knockout cells, are resistant to apoptosis and do not release cytochrome *c* from their mitochondria. Thus, mitochondrial signaling of apoptosis requires either Bak, which usually resides in mitochondria, or Bax, which translocates to mitochondria after apoptotic signaling. The coexistence of Bax and Bak in cells provides a redundancy in the signaling pathway. After tBid translocation to mitochondria, Bax and Bak are postulated to form large pores that release cytochrome *c* and other proteins across the outer membrane.

Other evidence strongly points to a role of inner membrane permeabilization and specifically the MPT in apoptotic signaling. When isolated nuclei and mitochondria are combined in a cell-free system, onset of the MPT induces the release of soluble factors from mitochondria that initiates apoptotic nuclear changes (101). Release of these factors occurs when large-amplitude mitochondrial swelling following the MPT causes rupture of the outer membrane. Direct evidence that the MPT occurs *in situ* in living cells comes from confocal microscopic experiments with hepatocytes stimulated with TNFα and Fas ligation. Following these stimuli, the normally impermeant green fluorophore calcein moves into mitochondria from the cytosol (9, 38). This inner membrane permeabilization precedes cytochrome *c* release, caspase 3 activation, and apoptotic cell death. Moreover, CsA blocks inner membrane permeabilization after TNFα and Fas ligation, confirming that inner membrane permeabilization occurs from onset of the MPT. Furthermore, CsA also inhibits cytochrome *c* release, caspase 3 activation, and apoptosis, which implicates the MPT as an essential step of apoptotic signaling in these models. Indeed, most factors promoting the MPT are proapoptotic and may mediate apoptotic signaling in many systems, including increased Ca^{2+}, ROS, pyridine nucleotide and glutathione oxidation, arachidonic acid, and GD3 ganglioside (for review, see 5). However, other slower proapoptotic signaling pathways coexist with the mitochondrial pathway, because in the presence of CsA, apoptosis in hepatocytes may be delayed rather than prevented. During delayed apoptosis in the presence of CsA, cell killing occurs without mitochondrial permeabilization, depolarization, and cytochrome *c* release, although caspase 3 becomes activated. Such apoptotic signaling that bypasses mitochondria is classified a type 1 pathway and may involve exaggerated DISC formation and activation of caspase 8, whereas apoptotic signaling requiring mitochondrial changes is a type 2 pathway (84).

An ongoing controversy concerns the two major mechanisms proposed to account for cytochrome *c* release after death receptor activation. These mechanisms seem mutually exclusive, but are both supported by strong and convincing experimental data. In the first mechanism, tBid interaction with Bax or Bak causes formation of pores in the mitochondrial outer membrane big enough to release cytochrome *c* and even larger proteins from the intermembrane space. In the second mechanism, death receptor activation causes PT pore opening in the inner membrane, leading to mitochondrial swelling, outer membrane rupture, and cytochrome *c* release, a mechanism supported by observations that CsA-sensitive inner membrane permeabilization precedes cytochrome *c* release, but contradicted by reports of cytochrome *c* release without mitochondrial depolarization or swelling (see 53). Although mitochondrial death receptor signaling leading to cytochrome *c* release and caspase 9 and 3 activation may just be different in different cell types, some hypotheses have been advanced to reconcile the two mechanisms. For example, Bax and other proapoptotic Bcl-2 family members may help form or regulate PT pores, and a pore with some of the properties of the PT pore has been reconstituted from Bax, the ANT, and other components (11). Moreover, recombinant Bax and Bid are reported to induce the MPT in isolated mitochondria (64, 76, 102), but these findings have not been universally reproduced (43, 47).

Thus, the question remains open as to how cytochrome *c* escapes mitochondria during apoptosis.

THE PERMEABILITY TRANSITION CAN OPERATE AN ATP SWITCH BETWEEN NECROTIC AND APOPTOTIC CELL DEATH

Apoptosis is an active process requiring ATP (25, 65). For example, caspase 9 activation by the cytochrome *c*/APAF-1 complex requires hydrolysis of ATP or dATP (58, 59). By contrast, necrotic cell death is the consequence of ATP depletion (68). By uncoupling mitochondria and activating the mitochondrial ATPase, onset of the MPT can lead to profound ATP depletion and necrosis. However, if the MPT develops heterogeneously and progresses slowly in mitochondria within a cell, then ATP levels will not become fully depleted, allowing apoptosis to develop (Fig. 7). Conversely, so-called secondary necrosis often develops in apoptotic cells, which may occur as mitochondrial injury becomes more severe and ATP levels fall below a critical level of ~10–15% of normal.

Necrotic cell death can be converted to apoptosis by preserving ATP levels. For example, hepatocytes subjected to ischemia/reperfusion or exposed to the calcium ionophore, Br-A23187, rapidly undergo ATP-dependent necrotic cell killing mediated by the MPT (46, 81). The importance of ATP depletion is illustrated by the ability of glycolytic ATP generation to rescue the hepatocytes from necrotic killing. However, ATP rescue does not prevent onset of the MPT. Rather after ATP rescue, hepatocytes undergo an MPT and caspase 3-dependent apoptosis. This apoptosis is blocked by CsA and thus is specifically caused by onset of the MPT. These experiments illustrate how the MPT can induce either necrotic cell death from ATP depletion or apoptosis from cytochrome *c* release and caspase activation (Fig. 7).

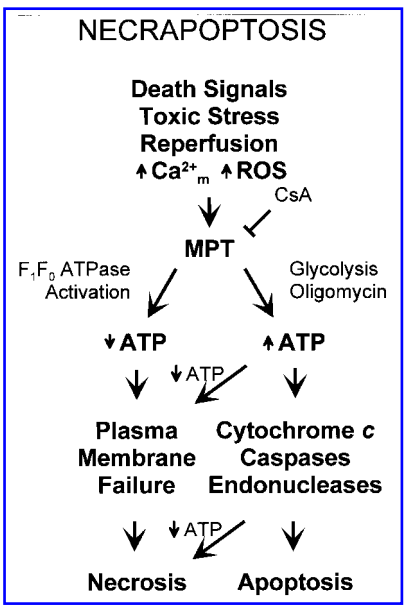

FIG. 7. Necrapoptosis and the switch between necrotic and apoptotic cell death. A variety of death signals and stresses promote onset of the MPT. When mitochondrial permeabilization occurs abruptly, the mitochondrial F_1F_0 ATPase becomes activated and causes ATP depletion, which leads to plasma membrane failure and necrotic cell death. If mitochondrial permeabilization progresses more slowly, glycolysis maintains ATP levels. In the presence of ATP, cytochrome *c* released after mitochondrial swelling following the MPT can activate cytosolic caspases, causing apoptotic rather than necrotic cell death. During the progression of apoptosis, ATP depletion may supervene to induce secondary necrosis.

Although necrosis and apoptosis have long been considered totally distinct phenomena, apoptotic and necrotic features often coexist after ischemia/reperfusion, viral infection, and exposure to toxic chemicals (83, 88). For example, injection of anti-Fas antibody into mice causes massive apoptosis, but this injury is also associated with fulminant hepatic failure, disruption of liver architecture, enzyme release, and liver inflammation, features usually associated with necrosis (73). Moreover, pharmacological inhibition of apoptosis prevents these necrotic and inflammatory changes (77). Indeed, vigorous controversies have developed as to whether cell killing in a particular setting is apoptosis or necrosis (30, 32, 74), because conventional distinctions between apoptotic and necrotic cell death can become lost in pathological situations. To emphasize death processes that begin with common signals and stresses, progress through shared pathways, such as mitochondrial permeabilization, and culminate in either cell lysis (necrotic cell death) or programmed cellular resorption (apoptosis), the term necrapoptosis has been introduced (52). In necrapoptosis, pure apoptosis and pure necrosis are extremes in a spectrum of changes in response to stresses and death signals, but the more common pathophysiological response is a mixture of features associated with both apoptotic and necrotic cell death.

POSSIBLE ROLE OF THE MITOCHONDRIAL PERMEABILITY TRANSITION IN MITOCHONDRIAL AUTOPHAGY

Although accumulating evidence indicates that the MPT plays a major role in both apoptotic and necrotic cell death, the question remains as to whether the MPT is physiologically important in cells that are not dying. One possible role of the MPT in nondying cells is for initiation of mitochondrial autophagy. Autophagy is the process by which cells degrade damaged and superfluous organelles, including mitochondria. In autophagy, lamellae of endoplasmic reticulum envelop a mitochondrion or other targeted organelle to create double-walled structures called autophagosomes that quickly acidify. Autophagosomes then acquire acid hydrolases by fusion with primary lysosomes, and their contents are ultimately digested and sometimes exocytosed (48). In liver, autophagy reclaims nutrients during fasting stimulated, in part, by glucagon (3, 51). Similarly in cultured hepatocytes, glucagon and nutrient deprivation stimulate autophagy, whereas incubation in serum- and insulin-containing growth medium inhibits autophagy (86, 87).

The signals targeting individual mitochondria for autophagy are not known, but recent evidence suggests that

onset of the MPT is an important event providing such signals. When cultured rat hepatocytes are exposed to serum deprivation and glucagon, spontaneous depolarization of individual mitochondria increases fivefold to ~1% per hour (26). CsA blocks this depolarization after autophagic stimulation. Depolarized mitochondria subsequently move into acidic autophagosomes that likewise increase in number by severalfold in a CsA-sensitive fashion. Thus, after autophagic stimulation, the MPT appears to initiate mitochondrial depolarization and subsequent sequestration into autophagosomes. In this way, the MPT represents a physiological mechanism that maintains cellular health.

By strategically targeting weak and injured mitochondria for autolysosomal degradation, the MPT averts cellular injury due to mitochondrial ROS formation and futile ATP consumption after respiratory inhibition and/or uncoupling. Excessive onset of the MPT, however, may overwhelm the autophagic apparatus and begin to induce apoptosis. Indeed, autophagy often accompanies apoptosis, as observed with a broad range of apoptotic inducers, including TNFα, serum/growth factor deprivation, and staurosporin and other toxins (17, 41, 75, 79, 82, 98). 3-Methyladenine, a specific inhibitor of autophagy, blocks TNFα-induced apoptosis in T-lymphoblastic leukemia cells and in other apoptotic models where autophagy is a prominent feature (14, 41, 82). Thus, the MPT may initiate a progressively stronger cellular response (57). Onset of the MPT limited to a relatively few mitochondria elicits the removal and eventual replacement by mitochondrial biogenesis of damaged and dysfunctional mitochondria. After greater involvement of mitochondria in the MPT, autophagy begins to be replaced by apoptosis, and with still greater involvement necrotic cell death from ATP depletion ensues. In this way, a continuum of changes may be elicited from involvement of a single basic event—mitochondrial inner membrane permeabilization via the MPT.

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ABBREVIATIONS

AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocator; APAF-1, apoptosis-inducing factor-1; CsA, cyclosporin A; CypD, cyclophilin D; DISC, death-inducing signaling complex; H_2O_2, hydrogen peroxide; MDCK, Madin–Darby canine kidney; MPT, mitochondrial permeability transition; $O_2^{\cdot -}$, superoxide; PT, permeability transition; ROS, reactive oxygen species; tBid, truncated Bid; *t*-BuOOH, *tert*-butyl hydroperoxide; TNFα, tumor necrosis factor-α; ΔpH, pH gradient; ΔΨ, electrical potential gradient.

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Address reprint requests to:
Dr. John J. Lemasters
Department of Cell and Developmental Biology
University of North Carolina at Chapel Hill
Room 236 Taylor Hall
Chapel Hill, NC 27599.7090

E-mail: Lemaster@med.unc.edu

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