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Mitochondrial Dysfunction in the Pathogenesis of Necrotic and Apoptotic Cell Death

John J. Lemasters,[1,8] Ting Qian,[1] Cynthia A. Bradham,[2,3] David A. Brenner,[2,3] Wayne E. Cascio,[2] Lawrence C. Trost,[4] Yoshiya Nishimura,[5] Anna-Liisa Nieminen,[6] and Brian Herman[7]

Mitochondria are frequently the target of injury after stresses leading to necrotic and apoptotic cell death. Inhibition of oxidative phosphorylation progresses to uncoupling when opening of a high conductance permeability transition (PT) pore in the mitochondrial inner membrane abruptly increases the permeability of the mitochondrial inner membrane to solutes of molecular mass up to 1500 Da. Cyclosporin A (CsA) blocks this mitochondrial permeability transition (MPT) and prevents necrotic cell death from oxidative stress, Ca^{2+} ionophore toxicity, Reye-related drug toxicity, pH-dependent ischemia/reperfusion injury, and other models of cell injury. Confocal fluorescence microscopy directly visualizes onset of the MPT from the movement of green-fluorescing calcein into mitochondria and the simultaneous release from mitochondria of red-fluorescing tetramethylrhodamine methylester, a membrane potential-indicating fluorophore. In oxidative stress to hepatocytes induced by *tert*-butylhydroperoxide, NAD(P)H oxidation, increased mitochondrial Ca^{2+}, and mitochondrial generation of reactive oxygen species precede and contribute to onset of the MPT. Confocal microscopy also shows directly that the MPT is a critical event in apoptosis of hepatocytes induced by tumor necrosis factor-α. Progression to necrotic and apoptotic cell killing depends, at least in part, on the effect the MPT has on cellular ATP levels. If ATP levels fall profoundly, necrotic killing ensues. If ATP levels are at least partially maintained, apoptosis follows the MPT. Cellular features of both apoptosis and necrosis 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.

KEY WORDS: Apoptosis; confocal microscopy; cyclosporin A; cytochrome *c*; ischemia/reperfusion; mitochondrial permeability transition; necrapoptosis; necrosis; oxidative stress.

INTRODUCTION

Life of higher multicellular organisms depends critically on the availability of oxygen. Mitochondria

[1] Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27799-7090.
[2] Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27799-7090.
[3] Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27799-7090.
[4] Department of Toxicology, Triangle Pharmaceuticals, Durham, North Carolina 27707.
[5] Division of Gastroenterology, Osaka National Hospital, Osaka, Japan.
[6] Department of Anatomy, Case Western University, Cleveland Ohio 44106.
[7] Department of Cellular and Structural Biology, The University of Texas Health Center at San Antonio, San Antonio, Texas 78285.
[8] Author to whom all correspondence should be sent (email: lemaster@med.unc.edu).

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are the principal site of cellular oxygen consumption whose purpose is the generation of ATP by oxidative phosphorylation. In highly aerobic tissues like heart, brain, and liver, mitochondria occupy up to 25% of cell volume. Given the importance of mitochondria for cell life, it comes as no surprise that mitochondrial dysfunction and failure leads to cell death. Indeed, much evidence indicates mitochondria are the main target of injury after stresses leading to necrotic and apoptotic cell death.

MITOCHONDRIA AND NECROTIC CELL DEATH

Necrosis

Necrotic cell death is the typical consequence of severe acute cellular injury, such as occurs in strokes and heart attacks (Trump *et al.*, 1995). The cellular events leading up to necrotic cell death are somewhat variable from one cell type to another, but certain events occur regularly. One of the earliest changes after oxygen deprivation or metabolic inhibition is the formation of protrusions of the plasma membrane called blebs (Lemasters *et al.*, 1983). These blebs contain cytosol and endoplasmic reticulum but generally exclude larger organelles like mitochondria. Cellular swelling of 30 to 50%, dilatation of cisternae of endoplasmic reticulum, and moderate mitochondrial swelling accompany this bleb formation.

To this point, cell injury is reversible. Irreversible injury occurs when one of the plasma membrane blebs ruptures (Lemasters *et al.*, 1987; Nieminen *et al.*, 1988). This all-or-nothing event leads to failure of the plasma membrane permeability barrier, release of intracellular enzymes and metabolites, and collapse of all electrical and ionic gradients across the plasma membrane. In hepatocytes, myocytes, and sinusoidal endothelial cells, a metastable state precedes rupture of the plasma membrane (Nieminen *et al.*, 1988; Gores *et al.*, 1989; Herman *et al.*, 1988). At the beginning of this metastable state, mitochondria depolarize and undergo the mitochondrial permeability transition (MPT; see below). Subsequently, lysosomes disintegrate, blebs coalesce and increase in size, cell swelling accelerates, and low molecular weight anionic fluorophores begin to leak from the cytosol (Zahrebelski *et al.*, 1995). This metastable state culminates with physical rupture of the plasma membrane and consequent equilibration of intracellular and extracellular contents.

In cultured sinusoidal endothelial cells, accelerated swelling and growth of blebs in the metastable state seem initiated by opening of a so-called death channel in the plasma membrane that is selectively permeable to anions, including organic anions up to a molecular weight of at least 600 Da (Nishimura and Lemasters, 1997). The cytoprotective amino acid, glycine, inhibits the death channel. After opening of the death channel, colloid osmotic forces and entry of cations (K^+ and Na^+) through open cation channels (Carini *et al.*, 1997; Ju *et al.*, 1994; Haddad and Jiang, 1994) drive the rapid swelling associated with the metastable state. Swelling continues until the plasma membrane ruptures, at which time the driving force for swelling is lost and further volume growth ceases abruptly. Since the permeability transition in mitochondria and membrane breakdown in lysosomes precede onset of the metastable state, a hydrolytic enzyme or factor released or activated by these organelles may be important for death channel opening. Opening of a glycine-sensitive plasma membrane channel of somewhat different properties has also been independently described in renal cells preceding necrotic cell death (Dong *et al.*, 1998).

Mitochondrial Dysfunction in Necrotic Cell Killing

The role of mitochondrial dysfunction in cytotoxicity leading to necrotic cell death can be assessed experimentally by the ability of glycolytic substrates to rescue cells from lethal cell injury (Nieminen *et al.*, 1990). As an alternative ATP source, glycolysis partially replaces ATP production lost after mitochondrial injury. This ATP, which can be as little as 15 or 20% of normal levels, rescues cells from necrotic death. Glucose is the prototypic glycolytic substrate and prevents anoxic cell killing in most cell types. However, the liver is different, since its function is to maintain blood glucose levels constant. Because of the absence of hexokinase, glucose is glycolyzed poorly by hepatocytes even during anoxia. Fructose is a much better glycolytic substrate, and fructose, but not glucose, prevents loss of viability of hepatocytes after anoxia, cyanide, and oligomycin (Nieminen *et al.*, 1990; Anundi *et al.*, 1987).

In aerobic hepatocytes, fructose at high concentrations causes intracellular ATP and P_i concentrations

to decrease because of ATP consumed in the fructokinase reaction and the accumulation of sugar phosphate metabolic intermediates (Mayes, 1993). The decline of ATP is often interpreted as evidence for fructose toxicity, but fructose-treated hepatocytes and livers maintain their ATP/ADP-P_i ratio since the decrease of ATP is offset by a decrease of P_i. The ATP/ADP-P_i ratio or phosphorylation potential, rather than ATP concentration, ATP/ADP ratio or energy charge, is the relevant thermodynamic variable reflecting cellular bioenergetic status (Nicholls and Ferguson, 1992). In anoxic livers and hepatocytes, fructose increases ATP and prevents hypoxic damage (Anundi *et al.*, 1987; Nieminen *et al.*, 1994). ATP does not need to increase to normal levels for cytoprotection to occur, since only a fraction of normal ATP is sufficient to prevent onset of necrotic cell death. Fructose also prevents hepatocellular toxicity caused by oxidant chemicals, indicating that mitochondria are a primary target of cytotoxicity in oxidative stress (Nieminen *et al.*, 1990; Imberti *et al.*, 1993).

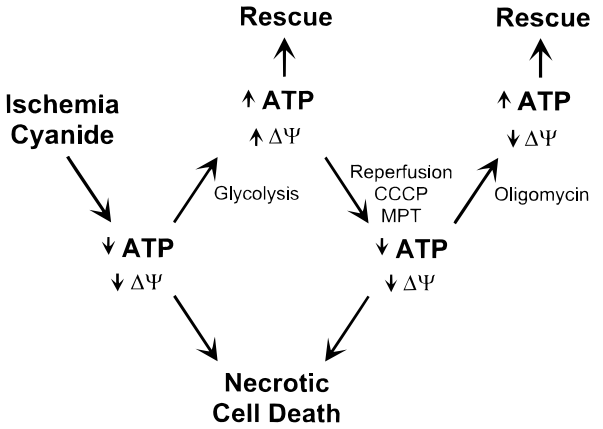

Fig. 1. Progression of mitochondrial injury during hypoxic and toxic injury. Respiratory inhibition during ischemia, hypoxia, or exposure to toxicants like cyanide cause mitochondrial depolarization and cellular ATP depletion. Glycolysis reverses this ATP depletion. The mitochondrial F_1F_0 ATP-synthase operating in the reverse mode utilizes some of the glycolytic ATP to restore mitochondrial $\Delta\Psi$. Uncoupling of mitochondria by the MPT after reperfusion or from protonophoric uncouplers like CCCP collapses $\Delta\Psi$, stimulates the mitochondrial F_1F_0-ATPase and abolishes glycolytic cytoprotection. Oligomycin inhibits the mitochondrial ATPase and prevents the futile uncoupler-stimulated ATP hydrolysis, which restores ATP and rescues cells without recovery of mitochondrial $\Delta\Psi$. Adapted from Nieminen *et al.*, 1994.

Mitochondrial Uncoupling in Necrotic Cell Killing

When cellular stress causes uncoupling of oxidative phosphorylation, the mitochondrial F_1F_0-ATPase becomes activated. This ATPase consumes ATP generated by glycolysis, so that glycolysis is no longer adequate to protect against cell killing. The importance of the uncoupler-stimulated mitochondrial ATPase is documented by the fact that inhibition of the mitochondrial ATPase with oligomycin protects cells against cytotoxicity when uncoupling is involved (Nieminen *et al.*, 1990, 1994; Imberti *et al.*, 1993). Oligomycin cytoprotection requires that a glycolytic substrate be present. Indeed, in the absence of glycolysis, oligomycin is itself toxic. Thus, fructose protects hepatocytes against cyanide, oligomycin, and anoxia, which inhibit but do not uncouple oxidative phosphorylation. Fructose does not prevent the cytotoxicity of classical protonophoric uncouplers like CCCP. Rather, the combination of fructose plus oligomycin is needed to prevent necrotic cell death.

These experiments define a progression of increasing mitochondrial injury (Fig. 1). Simple respiratory inhibition by anoxia or cyanide causes an inhibition of oxidative phosphorylation, collapse of mitochondrial membrane potential ($\Delta\Psi$), ATP depletion, and, ultimately, necrotic cell death. Addition of fructose partially restores ATP by glycolysis and rescues cells from necrotic killing. The mitochondrial F_1F_0-ATP synthase operating in the reverse mode utilizes some of the glycolytic ATP to restore mitochondrial $\Delta\Psi$, although oxidative phosphorylation remains inhibited. When mitochondrial uncoupling supervenes, such as after addition of CCCP or onset of the MPT, then the uncoupler-stimulated F_1F_0-ATPase is activated to hydrolyze ATP made available by glycolysis. Consequently, ATP levels fall profoundly, the mitochondria depolarize, and lethal cell injury follows. The specific involvement of the ATPase is documented experimentally by the ability of oligomycin, an inhibitor of the F_1F_0-ATPase, to prevent uncoupler-induced ATP depletion and subsequent cell death. However, oligomycin does not restore mitochondrial $\Delta\Psi$.

Other ionophores produce cytotoxicity by mitochondrial uncoupling. In particular, the calcium ionophore Br-A23187, often used as a model of Ca^{2+}-dependent cytotoxicity, causes necrotic killing of hepatocytes that is prevented by fructose in combination with oligomycin (Nieminen *et al.*, 1990; Qian *et al.*, 1999a). By this same criterion, moderate doses of oxidant chemicals, such as *tert*-butylhydroperoxide

(TBH), also induce cytotoxicity mediated by mitochondrial uncoupling (Imberti, 1993).

MITOCHONDRIAL PERMEABILITY TRANSITION

Permeability Transition Pore

The mitochondrial permeability transition (MPT) is a reversible phenomenon by which mitochondria become freely permeable to solutes of molecular mass less than about 1500 Da (Bernardi, 1996). Ca^{2+}, P_i, reactive oxygen species (ROS), and numerous oxidant chemicals induce the MPT, whereas Mg^{2+}, low pH, and cyclosporin A block the MPT. After onset of the MPT, mitochondria depolarize, uncouple, and undergo large amplitude swelling. By patch clamping, a cyclosporin A-sensitive permeability transition pore (PTP) was identified in the mitochondrial inner membrane whose opening is responsible for onset of the MPT (Szabo and Zoratti, 1991). Conductance through the PTP is so great that opening of a single PTP may be sufficient to cause mitochondrial depolarization and swelling (Zoratti and Szabo, 1995).

The molecular composition of the PTP is the subject of ongoing investigation. The PTP is likely comprised, at least in part, by the adenine nucleotide translocator (ANT) protein, since specific ANT ligands, atractyloside and bongkrekic acid, modulate PTP conductance (Halestrap and Davidson, 1990). Moreover, PTP activity has been reconstituted by inserting purified ANT into black lipid bilayer membranes and liposomes (Brustovetsky and Klingenberg, 1996). Other proteins may also associate to form the pore complex. These proteins include cyclophilin D (a cyclosporin A-binding protein) in the matrix, creatine kinase in the intermembrane space, porin and hexokinase in the outer membrane, and the proapoptotic protein, bax (Beutner *et al.*, 1996; Marzo *et al.*, 1998). The admixture of proteins from the matrix, inner membrane, intermembrane space, and outer membrane suggests that the PTP must span the inner and outer membrane, presumably at Hackenbrock's contact sites (Hackenbrock, 1968). However, PTP activity has also been reported in mitochondrial membranes from triple ANT knockout yeast (Lohret *et al.*, 1996). This finding implies that the ANT may not be an obligatory component of the pore complex. Other evidence suggests that the PTP is involved in the importation into mitochondria of polypeptides synthesized on cytosolic ribosomes (Lohret *et al.*, 1997).

Protection by Cyclosporin A Against Necrotic Cell Death

Soon after the discovery that cyclosporin A inhibits the PTP, reports began to appear that cyclosporin A blocks necrotic cell killing from oxidative stress, anoxia, ischemia/reperfusion, and a variety of toxic chemicals (Imberti *et al.*, 1990, 1992, 1993; Nazareth *et al.*, 1991; Broekemeier *et al.*, 1992; Kass *et al.*, 1992; Snyder *et al.*, 1992; Griffiths and Halestrap, 1993; Pastorino *et al.*, 1993; Fujii *et al.*, 1994). However, cytoprotection by cyclosporin A does not prove the involvement of the PTP in cytotoxicity. Cyclosporin A has other potent pharmacological actions, most importantly an immunosuppressive effect mediated by inhibition of calcineurin, a protein phosphatase involved in T cell activation (Halloran, 1996). A legitimate question was whether the MPT could occur at all within intact cells, since free Mg^{2+} in the cytosol of healthy cells is 0.5 m*M* or greater (Raju, *et al.*, 1989), a concentration that strongly inhibits the MPT in isolated mitochondria. Indeed, when cells are injured and ATP levels decrease, Mg^{2+} chelated by ATP is released and free Mg^{2+} actually increases (Herman *et al.*, 1990). In addition, pH falls and ADP increases after cell injury, and both acidic pH and elevated ADP block the MPT.

Confocal Microscopy of the Mitochondrial Permeability Transition in Individual Mitochondria within Living Cells

To address the question of whether the MPT actually occurs as cells respond to injurious stresses, laser scanning confocal microscopy was used to visualize membrane permeability of individual mitochondria within cells (Nieminen *et al.*, 1995). Confocal microscopy creates submicron optical slices that resolve individual mitochondria in three dimensions within thick cells and tissues. When cultured rat hepatocytes or rabbit cardiac myocytes are incubated with calcein acetoxymethyl (AM) ester, the neutral ester diffuses across the plasma membrane. Inside the cytosol, esterases hydrolyze calcein-AM to its pentavalent free acid form, which is trapped in the cytosol. Confocal microscopy shows the diffuse distribution of calcein through-

out the cytosol and nucleus (Fig. 2). Within the diffuse cytosolic fluorescence are numerous dark round and oval voids of about a micron in diameter. Each void represents one mitochondrion, as shown directly by colabeling with red-fluorescing tetramethylrhodamine methylester (TMRM), a cationic fluorophore that accumulates in mitochondria in response to the highly negative mitochondrial membrane potential. The dark mitochondrial voids within the calcein fluorescence persist for many hours and reflect the fact that the mitochondrial inner membrane is highly impermeable to this 623-Da solute. Importantly, the voids are present before TMRM is added (Lemasters *et al.*, 1998; Qian *et al.*, 1999b). Thus, quenching or other interaction between calcein and TMRM is not responsible for the dark voids, as has been claimed (Petronilli *et al.*, 1999).

In many models of cell injury, green cytosolic calcein fluorescence redistributes relatively abruptly into the mitochondria of individual cells after a latency of minutes or hours after onset of an injurious stress. Simultaneously, mitochondrial lose TMRM fluorescence. These events indicate an increase in nonspecific permeability of the mitochondrial inner membrane and a collapse of the mitochondrial membrane potential, respectively. Since blockers of the MPT, like cyclosporin A, prevent both the movement of calcein into the mitochondrial matrix and mitochondrial depolarization, it is evident that these events represent onset of the MPT *in situ*.

Although onset of the MPT always causes collapse of the mitochondrial membrane potential, loss of membrane potential does not always mean onset of the MPT. For example, after exposure of hepatocytes to the protonophoric uncoupler, CCCP, mitochondria rapidly lose their TMRM fluorescence, but the mitochondrial voids in the calcein fluorescence persist (Nieminen *et al.*, 1995). In the absence of an external stress, the mitochondria of rat hepatocytes and rabbit cardiac myocytes exclude calcein fluorescence for several hours (Nieminen *et al.*, 1995; Chacon *et al.*, 1996; Ohata *et al.*, 1998). Similarly, fluorophores loaded into mitochondria are retained for many hours (Qian *et al.*, 1999b; Trollinger *et al.*, 1997; Lemasters *et al.*, 1999). This suggests that the PTP of unstressed cells are closed virtually continuously, since even transient openings would lead to equilibration of calcein between the cytosol and the mitochondria

THE MITOCHONDRIAL PERMEABILITY TRANSITION IN NECROTIC CELL KILLING

pH-Dependent Ischemia/Reperfusion Injury

Ischemia is the loss of blood flow and, hence, oxygen supply to a tissue or organ. Anaerobic glycoly-

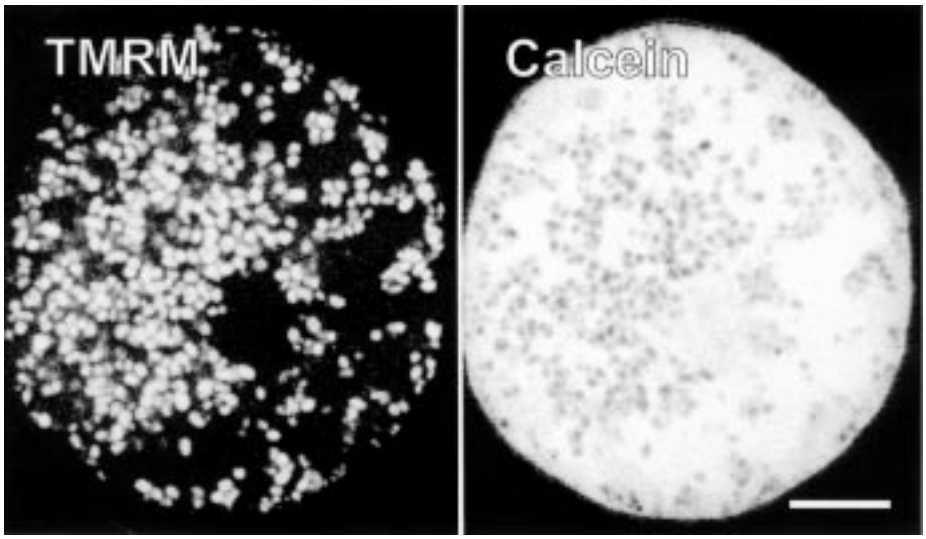

Fig. 2. Confocal imaging of mitochondrial membrane permeability and membrane potential. A rat hepatocyte was coloaded with red-fluorescing TMRM (left panel) and green-fluorescing calcein (right panel), and simultaneous red and green fluorescence images were collected by laser scanning confocal microscopy. Cationic TMRM is drawn electrophoretically into mitochondria in proportion to mitochondrial $\Delta\Psi$. Calcein enters the cytosol but is excluded from mitochondria, which appear as small round dark voids in the calcein image. Each mitochondrion in the red TMRM image has a corresponding dark void in the green calcein image. Bar is 10 μm.

sis, the hydrolysis of ATP and other nucleotides, and the release of protons from acidic organelles cause tissue pH to decrease in ischemia by more than a unit (Ch'en *et al.*, 1997; Gores *et al.*, 1989b; Bronk and Gores, 1991). This acidosis protects strongly against the onset of hypoxic and ischemic cell death (Penttila and Trump, 1974; Bonventre and Cheung, 1985; Gores *et al.*, 1988). Acidosis also dramatically delays cell killing by oxidant chemicals, ionophores, and alkylating agents (Nieminen *et al.*, 1990; Imberti *et al.*, 1993).

The naturally occurring acidosis of ischemia prevents onset of cell death, but reperfusion after ischemia can worsen cell injury and precipitate tissue necrosis (Cryer, 1997). In cultured cells and perfused organs, anoxia at acidotic pH followed by reoxygenation at normal pH simulates the oxygen deprivation and acidosis of ischemia and the recovery of oxygen and pH after reperfusion. In this model, reperfusion causes loss of cell viability and release of intracellular enzymes like lactate dehydrogenase (Gores *et al.*, 1988; Currin *et al.*, 1991, 1996; Bond *et al.*, 1991, 1993; Harper *et al.*, 1993; Zager *et al.*, 1993; Kaplan *et al.*, 1995; Qian *et al.*, 1997; Nishimura *et al.*, 1998). Recovery of normal pH after reperfusion causes this injury, since reoxygenation at low pH prevents cell killing entirely, whereas restoration of normal pH without reoxygenation produces the same cell killing as restoration of pH with reoxygenation. The paradoxical worsening of injury upon restoration of normal pH after reperfusion of ischemic tissue is called the pH paradox.

Intracellular pH directly mediates cell killing in the pH paradox. Ionophores like monensin, which accelerate the increase of intracellular pH after reperfusion, also accelerate necrotic cell killing (Bond *et al.*, 1993). Conversely, inhibition of Na^+/H^+ exchange with dimethylamiloride or Na^+-free medium delays the increase of intracellular pH after reperfusion and prevents reperfusion-induced necrotic cell killing almost completely (Bond *et al.*, 1993; Harper *et al.*, 1993; Kaplan *et al.*, 1995; Qian *et al.*, 1997). pH-dependent cell killing occurs independently of changes of cytosolic and extracellular free Ca^{2+}, and acidic intracellular pH is cytoprotective when intracellular and extracellular Na^+ concentrations are equilibrated with monensin (Imberti *et al.*, 1990; Ch'en *et al.*, 1997; Bond *et al.*, 1993; Harper *et al.*, 1993; Qian *et al.*, 1997). Thus, secondary changes of intracellular Na^+ and Ca^{2+} do not mediate the strong effect of intracellular pH on necrotic cell killing.

Contribution of the PTP to the pH Paradox

pH below 7 strongly inhibits conductance through the PTP, and thus pH-dependent onset of the MPT might be responsible for pH-dependent reperfusion injury. In support of this hypothesis, cyclosporin A prevents pH-dependent cell killing after simulated ischemia/reperfusion to cultured rat hepatocytes (Qian *et al.*, 1997). Notably, cyclosporin A protects when added only during the reperfusion phase, demonstrating that cell killing is the specific consequence of reperfusion. Cyclosporin A also protects perfused hearts against anoxia/reoxygenation injury, an effect associated with prevention of redistribution of radioactively labeled sugar phosphates from the cytosolic space into the mitochondrial matrix space (Griffiths and Halestrap, 1993, 1995).

Confocal microscopy shows directly that onset of the MPT accompanies pH-dependent reperfusion injury (Qian *et al.*, 1997). In cultured hepatocytes, after anoxia for 4 h at pH 6.2 to simulate ischemia, the MPT fails to occur, since cytosolic calcein fluorescence is still excluded from mitochondria (Fig. 3). By contrast, mitochondrial depolarization does occur and mitochondria lose virtually all their red fluorescent staining with TMRM, which illustrates how mitochondrial depolarization can be independent of onset of the MPT. When these hepatocytes are reoxygenated at normal pH (simulated reperfusion), mitochondria begin to repolarize, as shown by reaccumulation of red-fluorescing TMRM. Subsequently, however, green-fluorescing calcein redistributes into the mitochondria from the cytosol, and TMRM is once again released. Increased mitochondrial membrane permeability and mitochondrial depolarization demonstrate directly onset of the MPT after reperfusion (Fig. 3). Several minutes after onset of the MPT, necrotic cell death occurs. In the few cells that do not undergo the MPT after reperfusion, viability is not lost. Moreover, reperfusion with acidotic buffer or in the presence of cyclosporin A, treatments that block conductance of the PTP, prevents increased mitochondrial membrane permeability, mitochondrial depolarization, and loss of cell viability. Thus, onset of the MPT causes pH-dependent cell killing in this model of ischemia/reperfusion injury.

Oxidative Stress

tert-Butylhydroperoxide (TBH) is a short-chain organic hydroperoxide, which is an analog of the lipid

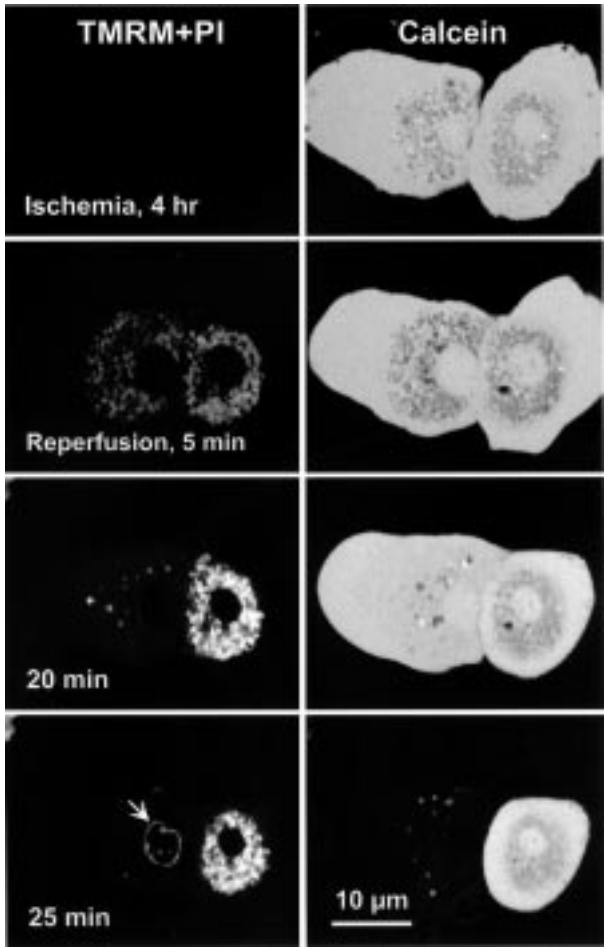

Fig. 3. Induction of the mitochondrial permeability transition by reperfusion after ischemia. Rat hepatocytes were loaded with TMRM and calcein. Red (TMRM, left panels) and green (calcein, right panels) fluorescence images were collected by confocal microscopy at the end of 4 h of simulated ischemia (anoxia at pH 6.2) and after 5, 20, and 25 minutes of reperfusion (oxygenated buffer at pH 7.4). Virtually all mitochondrial TMRM fluorescence was lost after 4 h of ischemia, indicating mitochondrial depolarization, whereas dark mitochondrial voids in the calcein fluorescence remained, indicating that the MPT had not occurred. After reperfusion, TMRM began to enter the mitochondria of both hepatocytes in the field within 5 min. After 20 min, one hepatocyte lost TMRM labeling, indicating mitochondrial depolarization. Simultaneously, the voids of calcein fluorescence filled in, indicating onset of the MPT. The hepatocyte subsequently lost viability, as indicated by loss of calcein fluorescence and nuclear uptake of propidium iodide after 25 min (arrow). In the other hepatocyte, TMRM continued to accumulate, and the dark voids in the calcein fluorescence persisted. Adapted from Qian *et al.*, 1997.

hydroperoxides formed during oxidative stress and ischemia/reperfusion. TBH causes oxidation of glutathione, NADH, and NADPH by the concerted action of glutathione peroxidase, glutathione reductase, and the mitochondrial NADP/NAD transhydrogenase (Sies and Moss, 1978). Addition of TBH to hepatocytes and other cells produces oxidative stress and necrotic cell death.

TBH is a potent inducer of the MPT in isolated mitochondria (Gunter and Pfeiffer, 1990). Similarly, in rat hepatocytes, TBH causes onset of the MPT, as visualized by confocal microscopy from calcein entry into mitochondria and loss of mitochondrial TMRM (Nieminen *et al.*, 1995). These cellular events are followed by ATP depletion and necrotic cell death. In isolated mitochondria, trifluoperazine blocks onset of the MPT (Broekemeier and Pfeiffer, 1989). Similarly, in hepatocytes exposed to TBH, trifluoperazine prevents calcein redistribution, TMRM release, ATP depletion, and cell death, which supports the conclusion that the MPT is responsible for TBH-induced cell killing (Imberti *et al.*, 1993; Harman *et al.*, 1990). Trifluoperazine does not prevent the cytotoxicity of CCCP, a protonophoric uncoupler that causes mitochondrial depolarization, ATP depletion, and cell death without inducing the MPT. Thus, protection by trifluoperazine is specific for MPT-mediated injury.

TBH causes rapid oxidation of mitochondrial pyridine nucleotides, visualized directly using confocal microscopy from the loss of mitochondrial autofluorescence excited with ultraviolet light (Nieminen *et al.*, 1997; Byrne *et al.*, 1999). After oxidation of mitochondrial NAD(P)H, mitochondrial free Ca^{2+} and generation of reactive oxygen species (ROS) increase sharply, as visualized by the Ca^{2+}-indicating fluorophore, Rhod-2 (Fig. 4) and the ROS-dependent conversion within mitochondria of dichlorofluor*escin* to highly fluorescent dichlorofluor*escein* (Fig 5). Subsequently, the MPT occurs and cell viability is lost. β-Hydroxybutyrate increases mitochondrial NAD(P)H by the β-hydroxybutyrate dehydrogenase and transhydrogenase reactions and delays TBH-induced cell killing, whereas lactate, which increases cytosolic NADH via the lactate dehydrogenase reaction, does not protect. Thus, oxidation of mitochondrial pyridine nucleotides promotes onset of the MPT and cell killing in TBH cytotoxicity.

Mitochondrial ROS formation increases up to 15-fold in hepatocytes exposed to TBH (Fig. 5). ROS generation occurs after the initial rapid oxidation of mitochondrial NAD(P)H. Diphenylphenylenediamine (DPPD), a scavenger of free radicals, and desferal, an iron chelator, block increased ROS formation after TBH and prevent both onset of the MPT and necrotic cell death. Thus, mitochondrial ROS formation also

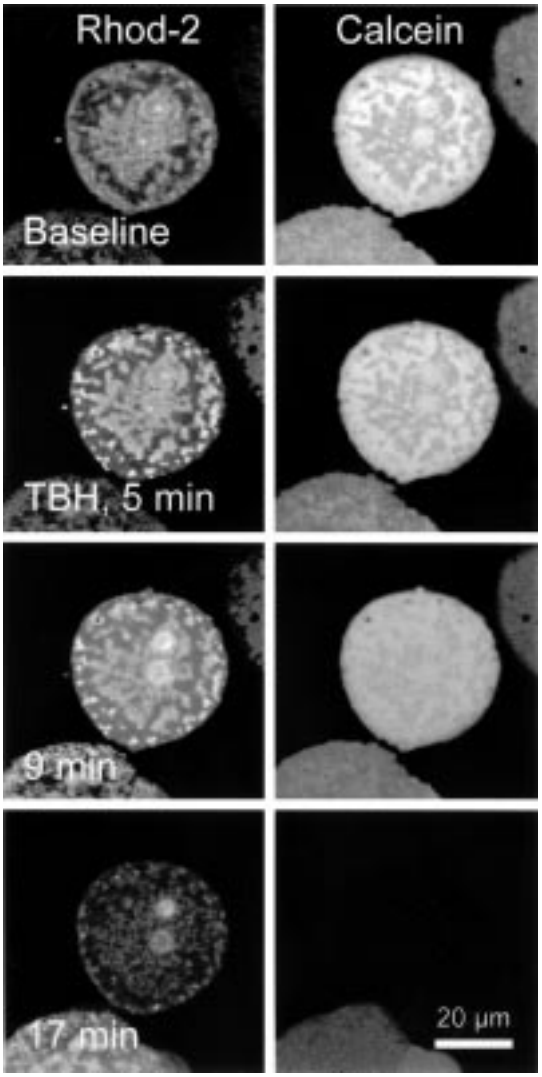

Fig. 4. Increased mitochondrial Ca^{2+} after exposure of hepatocytes to *t*-butylhydroperoxide. Cultured rat hepatocytes were coloaded with Rhod-2, a Ca^{2+} indicator, and calcein. Red Rhod-2 fluorescence (left panels) and green calcein fluorescence (right panels) were imaged by confocal microscopy. In the baseline image, Rhod-2 fluorescence was faint and calcein fluorescence was confined to the cytosol and nucleus, leaving mitochondria as dark voids. After exposure to 100 μM TBH, mitochondrial Rhod-2 fluorescence increased within 5 min with little change of calcein fluorescence. After 9 min, cytosolic Rhod-2 fluorescence began to increase, and mitochondria began to fill with calcein. After 17 min, viability was lost, and most Rhod-2 and calcein fluorescence was lost. Adapted from Byrne *et al.*, 1999.

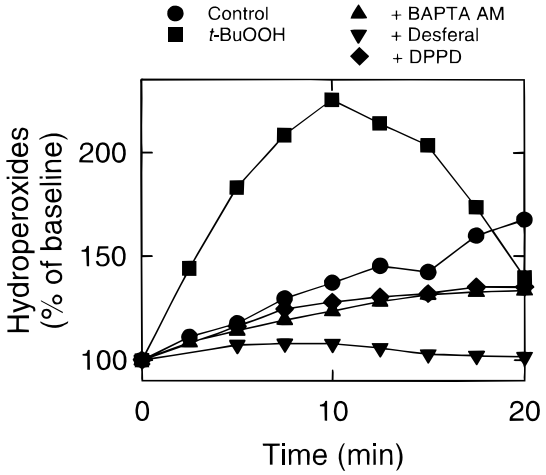

Fig. 5. Hydroperoxide formation after exposure of hepatocytes to *t*-butylhydroperoxide. Cultured rat hepatocytes were loaded with dichlorofluorescin, which reacts with hydroperoxides to form green-fluorescing dichlorofluorescein. After different treatments, green fluorescence was collected by confocal microscopy at various time points and quantified for individual cells from the digital images. Without additional treatment, hydroperoxide formation measured by dichlorofluorescein fluorescence increased about 40% in 10 min. After treatment with 100 μM TBH, hydroperoxide formation increased much more rapidly. Dichlorofluorescein fluorescence peaked after 10 min and subsequently declined as onset of the MPT and cell death occurred. Most hydroperoxide formation occurred in mitochondria both with and without TBH (see Nieminen *et al.*, 1997; Byrne *et al.*, 1999). BAPTA-AM, an intracellular Ca^{2+} chelator, diphenylphenylenediamine (DPPD), a free radical scavenger, and desferal, an inhibitor of iron-catalyzed free radical formation, each blocked mitochondrial hydroperoxide formation stimulated by TBH. The agents also blocked onset of the MPT and TBH-induced necrotic cell killing. Adapted from Nieminen *et al.*, 1997 and Byrne *et al.*, 1999.

promotes the MPT during oxidative stress with TBH. ROS may oxidize protein thiols to cause PTP opening, as shown in isolated mitochondria (Constantini *et al.*, 1996; Kowaltowski *et al.*, 1996). In neurons, mitochondrial ROS are generated during excitotoxic stress with glutamate and NMDA receptor agonists (Dugan *et al.*, 1995; Reynolds and Hastings, 1995) and several recent studies show that the MPT also mediates excitotoxic injury to neurons (Isaev *et al.*, 1996; Nieminen *et al.*, 1996; Schinder *et al.*, 1996; White and Reynolds, 1996).

Chelation of intramitochondrial Ca^{2+} with BAPTA AM prevents the increase of mitochondrial-free Ca^{2+} in hepatocytes exposed to TBH and completely blocks the stimulation of mitochondrial ROS generation (Fig. 5) (Byrne *et al.*, 1999). BAPTA AM does not prevent the rapid early oxidation of mitochondrial NAD(P)H, but does inhibit the late phase of more complete oxidation that is attributed to ROS generation. By comparison, inhibition of ROS formation with desferal delays, but does not block, the rise of mitochondrial Ca^{2+} after TBH.

These results support a model in which the initial effect of TBH is to oxidize mitochondrial NAD(P)H (Fig. 6). Pyridine nucleotide oxidation then disrupts mitochondrial Ca^{2+} homeostasis to cause an increase of mitochondrial Ca^{2+}, which, in turn, stimulates intramitochondrial ROS formation and onset of the MPT. ROS formation stimulated by the increase of mitochondrial Ca^{2+} may promote more mitochondrial Ca^{2+} uptake, either directly or by increasing NAD(P)H oxidation, thereby amplifying the signals promoting the MPT. Finally, onset of the MPT causes mitochondrial uncoupling, inhibition of mitochondrial ATP formation, accelerated ATP hydrolysis by the mitochondrial ATPase, and ATP depletion-dependent necrotic cell death.

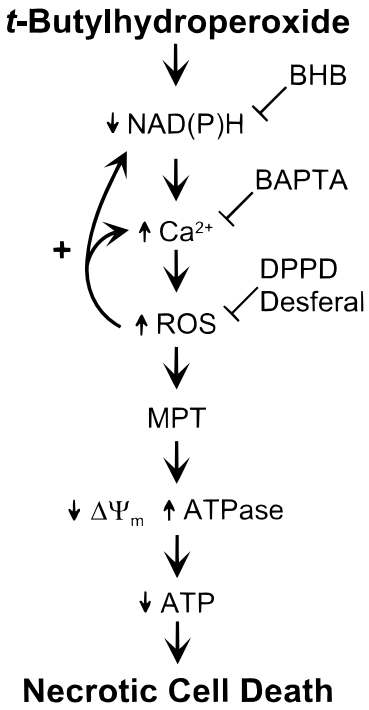

Fig. 6. Progression of mitochondrial changes leading to necrotic cell death during oxidant stress with *t*-butylhydroperoxide. *tert*-Butylhydroperoxide oxidizes mitochondrial NADH and NADPH. Mitochondrial Ca^{2+} homeostasis is then disrupted, and mitochondrial Ca^{2+} increases. Increased mitochondrial Ca^{2+} stimulates mitochondrial formation of reactive oxygen species (ROS), including hydroperoxides measured with dichlorofluorescein. Both increased Ca^{2+} and ROS formation promote onset of the MPT, which causes collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) and activation of mitochondrial ATPases. ATP depletion and necrotic cell death then ensue. Formation of ROS augments NAD(P)H oxidation and Ca^{2+} disregulation, which amplifies the signals promoting the MPT. β-Hydroxybutyrate (BHB) increases mitochondrial NAD(P)H and delays cell injury. Intracellular Ca^{2+} chelation with BAPTA blocks the increase of mitochondrial Ca^{2+}, which prevents subsequent ROS formation, onset of the MPT, and cell death. Desferal and diphenylphenylenediamine (DPPD) also inhibit ROS formation and prevent onset of the MPT and cell killing. Adapted from Byrne *et al.*, 1999.

Reye's Syndrome

Symptoms of Reye's syndrome include high fever, vomiting, fulminant hepatic failure and encephalopathy, advancing to coma and death in children after a prodromal viral illness (Heubi *et al.*, 1987). Electron microscopy of the liver and brain of Reye patients shows large-amplitude mitochondrial swelling. Suppression of ureagenesis and β-oxidation also implicates a specific mitochondrial injury. The pathogenesis of Reye's syndrome is associated with aspirin usage and the disease markedly declined after the U.S. Surgeon General advised against aspirin use in children. A Reye-related syndrome is also associated with other drugs and substances, particularly the pediatric use of the anticonvulsant, valproic acid (Gerber *et al.*, 1979).

The role of the MPT in Reye's syndrome is supported by the observation that salicylate induces a cyclosporin A-sensitive MPT in isolated rat liver mitochondria (Trost and Lemasters, 1996). Aspirin itself does not induce the MPT unless first hydrolyzed to salicylate, as occurs in the body after aspirin ingestion. Other substances implicated in Reye-related toxicity also induce cyclosporin A-sensitive onset of the MPT, including Neem oil and adipic, isovaleric, 3-mercaptopropionic, 4-pentenoic, and valproic acids. Although salicylate is a weak uncoupler, equivalent uncoupling with CCCP does not induce the MPT, and other MPT inducers, like valproic acid, produce negligible mitochondrial depolarization. Thus, membrane depolarization is not the mechanism by which Reye's-related chemicals promote the MPT.

Salicylate is also directly cytotoxic to cultured rat hepatocytes (Trost and Lemasters, 1997). This cytotoxicity is blocked by cyclosporin A and confocal microscopy shows onset of the MPT *in situ* from the movement of calcein from the cytosol into the mitochondria. Onset of the MPT in hepatocytes and loss of viability after salicylate are blocked by cyclosporin A and its nonimmunosuppressive analog, 4-methylvaline cyclosporin. Overall, these data strongly support the hypothesis that the MPT is involved in the pathophysiology of Reye-related drug toxicities.

The MPT in isolated mitochondria, induced by salicylate, does not occur unless a small amount of

Ca^{2+} is added (Trost and Lemasters, 1996). Similarly, high extracellular Ca^{2+} concentration promotes salicylate toxicity to cultured hepatocytes (Trost and Lemasters, 1997). The Ca^{2+} channel blocker, verapamil, decreases salicylate-induced killing of hepatocytes, an effect associated with decreased mitochondrial-free Ca^{2+} measured by confocal microscopy of Rhod-2. Synergism between salicylate and Ca^{2+} may explain the idiopathic nature of Reye's syndrome, since the vast majority of patients taking aspirin experience no toxicity. A prodromal viral illness, possibly in combination with a preexisting metabolic defect, may cause abnormal loading of mitochondria with Ca^{2+}. In such predisposed individuals, exposure to salicylate or other Reye-related toxicant precipitates onset of the MPT, producing a Reye-like illness. Onset of the MPT and resulting mitochondrial uncoupling would explain the clinical features of Reye's syndrome—hypoglycemia, hyperammonemia, elevation of dicarboxylic and free fatty acids, inhibition of β-oxidation, and large-amplitude mitochondrial swelling.

THE MITOCHONDRIAL PERMEABILITY TRANSITION IN APOPTOSIS

Apoptosis

A second mode of cell death is apoptosis. Apoptosis, unlike necrosis, is a form of physiological cell death that causes cell deletion without inflammation, scarring or release of cellular contents. As defined by Kerr, Wyllie and Currie (1972), apoptosis describes a process of controlled cell deletion that has an opposite role to mitosis in the regulation of cell populations. In apoptosis, individual cells separate from their neighbors and begin a characteristic sequence of structural and biochemical changes. These changes include cell shrinkage, alteration of plasma membrane lipids, condensation of chromatin, internucleosomal DNA degradation, and shedding of membrane-bound cytoplasmic fragments containing ultrastructurally intact organelles and chromatin. These apoptotic bodies are taken up by adjacent cells and macrophages. Specific signals, such as tumor necrosis factor-alpha (TNFα) and FAS ligand, can trigger apoptosis through a cascade of cysteine–aspartate proteases called caspases (Nunez *et al.*, 1998). In its purest form, apoptosis does not attract inflammatory cells (neutrophils and monocytes) or lead to scar formation.

The Mitochondrial Permeability Transition in Apoptosis

When purified nuclei and isolated mitochondria are combined in a cell-free system, onset of the MPT causes the release of soluble factors from mitochondria that activate caspases and initiate apoptotic nuclear changes (Susin *et al.*, 1998). These factors include cytochrome *c* and apoptosis-inducing factor (AIF) (Liu *et al.*, 1996; Susin *et al.*, 1999). Rupture of the outer membrane caused by large-amplitude mitochondrial swelling is the likely mechanism by which proapoptotic mitochondrial factors are released.

Cytochrome *c*, the best studied of these proapoptotic factors, binds to apoptosis-activating factor-1 (APAF-1) (Li *et al.*, 1997). Subsequent binding of ATP (or dATP) to this complex activates caspase 9, which, in turn, activates caspase 3. Activation occurs by proteolytic cleavage of the respective inactive procaspases. Activated caspase 3 then initiates the final execution stage of apoptosis, including PARP cleavage, internucleosomal DNA hydrolysis, cell shrinkage, chromatin margination, and nuclear lobulation. Another caspase (caspase 8), acts upstream of mitochondria. Binding of TNFα and FAS ligand to their receptors activates caspase 8, which cleaves bid, a member of the protooncogene bcl-2 family of proteins (Luo *et al.*, 1998; Li *et al.*, 1998). Bid then translocates to mitochondria to induce cytochrome *c* release. Other pro- and antiapoptotic members of the bcl-2 family also bind to mitochondria to promote or block, respectively, the MPT (Susin *et al.*, 1998).

Whether the MPT actually occurs inside intact cells during apoptosis has been controversial. Some studies conclude that release of cytochrome *c* during apoptosis occurs without mitochondrial depolarization and onset of the MPT (Kluck *et al.*, 1997; Yang *et al.*, 1997). However, onset of the MPT, as visualized from movement of calcein into mitochondria and simultaneous release of TMRM, does occur during apoptosis induced by TNFα in sensitized hepatocytes, beginning 8–9 h after TNFα treatment (Fig. 7) (Bradham *et al.*, 1998). This MPT precedes cytochrome *c* release, caspase-3 activation, and the execution stage of apoptosis. Cyclosporin A blocks the MPT induced by TNFα in hepatocytes and prevents cytochrome *c* release, caspase-3 activation, and apoptotic cell death (Fig. 8). Indeed, cyclosporin A can be added up to the time of onset of the MPT and still be cytoprotective. Thus, cyclosporin A can be added as late as 9 h after TNFα treatment and still prevent apoptosis.

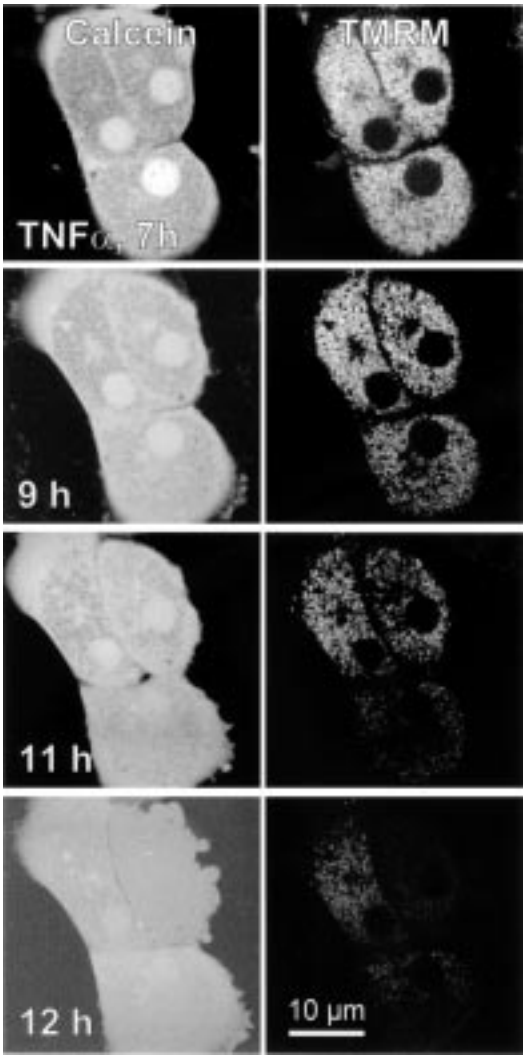

Fig. 7. The mitochondrial permeability transition in hepatocytes during TNFα-induced apoptosis. Rat hepatocytes were sensitized to TNFα with an adenovirus expressing an IκB super-repressor and loaded with calcein and TMRM. Green fluorescence (calcein, left panels) and red fluorescence (TMRM, right panels) were imaged by confocal microscopy after exposure to 30 ng/ml TNFα. After 7 h, mitochondria continued to retain TMRM and remained impermeable to calcein. Subsequently, TMRM began to be released from individual mitochondria. Simultaneously, these mitochondria took up calcein. After 12 h, virtually all mitochondria were depolarized and permeable to calcein. At this point, hepatocytes begin to shrink and undergo the zeiotic blebbing characteristic of apoptosis. Adapted from Bradham *et al.*, 1998.

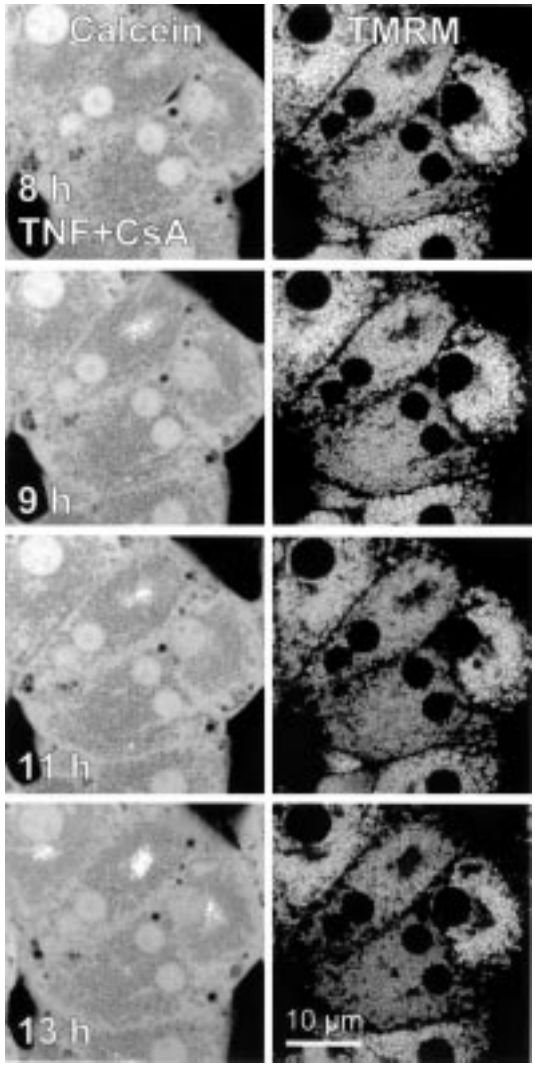

Fig. 8. Inhibition of the MPT and apoptosis by cyclosporin A. Sensitized hepatocytes were treated with 30 ng/ml TNFα in the presence of 2 μ*M* cyclosporin A and then loaded with TMRM and calcein for confocal microscopy, as described in Fig. 7. Cyclosporin A blocked mitochondrial uptake of calcein (left panels) and release of TMRM (right panels), and apoptosis did not occur. Adapted from Bradham *et al.*, 1998.

After about 9 h of exposure to TNFα, individual mitochondria begin to undergo the MPT, become permeable to calcein and depolarize. For the next few subsequent hours, polarized mitochondria coexist with depolarized mitochondria that have yet to undergo the MPT. The admixture of permeabilized and nonpermeabilized mitochondria may explain reports of cytochrome *c* release from cells still containing polarized mitochondria. Similarly, during staurosporin-induced apoptosis to PC6 pheochromocytoma cells, release of a transfected fusion protein of cytochrome *c* and green fluorescent protein (GFP) from mitochondria accompanies, but does not precede, mitochondria depolarization (Heiskanen *et al.* 1999). These findings support the hypothesis that mitochondrial swelling and rupture

of the outer membrane cause cytochrome *c* release during apoptosis. Thus, the MPT is at the middle of the apoptotic signaling cascade—upstream of cytochrome *c* release and activation of caspases-3 and-9, and downstream of receptor binding, caspase-8 and bid. However, in some models of apoptosis, signaling through mitochondria is bypassed and upstream receptor-linked caspases appear to activate caspase-3 directly (Scaffidi *et al.*, 1998).

What Determines Whether Apoptosis or Necrosis Occurs After the Mitochondrial Permeability Transition?

Apoptotic signaling requires ATP (Richter *et al.*, 1996; Leist *et al.*, 1997; Eguchi *et al.*, 1997). Specifically, activation of procaspase-9 by the cytochrome *c*/APAF-1 complex requires ATP or dATP (Liu *et al.*, 1996; Li *et al.*, 1997). By contrast, necrotic cell death is typically the consequence of ATP depletion. Indeed, glycolytic ATP generation prevents necrotic cell death in a wide variety of models (Nieminen *et al.*, 1990, 1994; Anundi *et al.*, 1987). Thus, the way the MPT affects cellular ATP levels may determine the mode of cell death that ensues. If the MPT causes ATP depletion, necrotic cell death occurs. However, if the MPT develops without severe ATP depletion, then apoptosis occurs instead.

Support for this concept comes from experiments with hepatocytes treated with Br-A23187, a Ca^{2+} ionophore. Br-A23187 treatment causes rapid onset of the MPT as visualized directly by confocal microscopy (Qian *et al.*, 1999a). Mitochondrial uncoupling caused by the MPT then leads to profound ATP depletion and necrotic cell death within an hour. The MPT and resulting cell death after Br-A23187 are blocked by cyclosporin A. Necrotic cell killing is also prevented when cellular ATP levels are maintained by fructose in the presence of oligomycin (see Fig. 1). However, the MPT still occurs in the presence of fructose plus oligomycin, and apoptosis instead of necrotic cell death develops several hours later. Cyclosporin A blocks this Br-A23187-dependent apoptosis.

Thus, the MPT plays a critical role in both necrosis and apoptosis. Necrosis develops when rapid onset of the MPT causes ATP depletion, whereas apoptosis develops when the MPT occurs without exhaustion of ATP (Fig. 9). A feature commonly observed in cells undergoing apoptosis is so-called secondary necrosis, in which necrotic cell killing with breakdown of the plasma membrane permeability barrier occurs as apoptosis is progressing. This secondary necrosis may develop as a consequence of ATP depletion because of mitochondrial failure during apoptosis (Fig. 9).

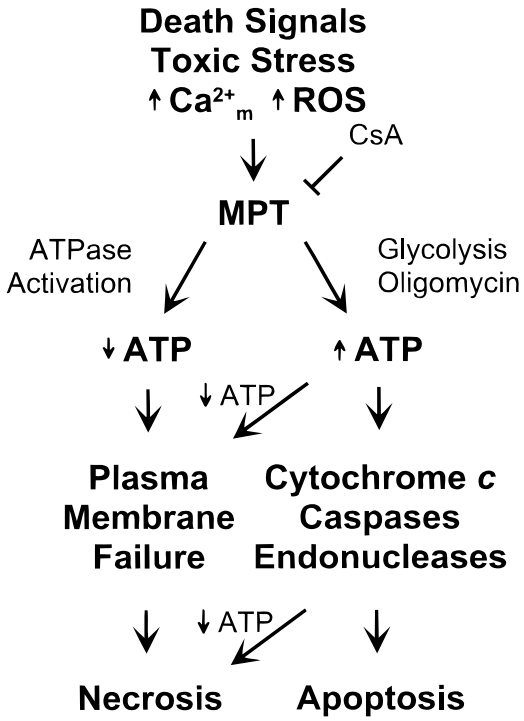

Fig. 9. Scheme showing the role of ATP in necrapoptosis mediated by the mitochondrial permeability transition. Death signals, toxic stresses, increased mitochondrial Ca^{2+}, and ROS formation all promote onset of the MPT. When the MPT occurs abruptly, activation of mitochondrial ATPases causes ATP depletion, which leads to plasma membrane rupture and necrotic cell death. If the mitochondrial ATPase is inhibited with oligomycin and a glycolytic substrate is available, or if the MPT progresses relatively slowly through the mitochondrial population of a single cell, then ATP levels remain relatively preserved even after onset of the MPT. Under such conditions, cytochrome *c* release activates a cascade of caspases, endonucleases, and other degradative enzymes, causing apoptotic rather than necrotic cell death. At any time, ATP depletion can supervene to cause secondary necrosis. Both apoptosis and necrosis are prevented by cyclosporin A. The term, necrapoptosis, describes such death processes that begin with common death inducers, progress by shared pathways, and culminate in either cell lysis (necrotic cell death) or programmed cellular resorption (apoptosis) depending on other factors, such as ATP.

Necrapoptosis

In tissue injuries due to ischemia/reperfusion, toxic chemicals, and viral infection, apoptotic and

necrotic features often coexist, which leads to controversies as to whether cell killing is apoptosis or necrosis (Grasl-Kraupp *et al.*, 1995; Ohno *et al.*, 1998). The nomenclature we use implicitly assumes that cell killing takes the form of either necrosis or apoptosis, phenomena assumed to be completely independent of one another. In fact, features of both modes of cell killing frequently occur not only in the same tissue but in the same cell. Moreover, the MPT represents a pathway that is shared by both apoptosis and necrosis. Recently, a new term, *necrapoptosis*, was introduced to emphasize death processes that begin with a common death signal or toxic stress, progress by shared pathways, but culminate in either cell lysis (necrotic cell death) or programmed cellular resorption (apoptosis) depending on other modifying factors (Lemasters, 1999). Lethal cell injury mediated by the MPT illustrates this concept (Fig. 9). Rapid onset of the MPT causes ATP depletion and early cell lysis. If the MPT progresses more slowly, such as after TNFα treatment, or if sources other than mitochondria are available for ATP generation, such as fructose, then profound ATP depletion is avoided, allowing the execution stage of apoptosis to proceed. However, if ATP collapses at a later stage, then cell lysis supervenes as secondary necrosis. In necrapoptosis, pure apoptosis and pure necrosis represent extremes in a continuous spectrum, and the more typical response to injurious stresses and other death signals is a mixture of events associated with apoptotic and necrotic cell death.

THERAPEUTIC IMPLICATIONS

The importance of understanding pathophysiologic mechanisms is the possibility to develop new therapeutic approaches. If the MPT is the key event causing or signaling both necrotic and apoptotic cell death, then pharmacological blockers of the PTP have the potential to prevent and even reverse disease. Thus, a drug like cyclosporin A might prevent both acute necrotic cell death, as occurs in ischemia/reperfusion and certain drug toxicities, and the more chronic apoptotic cell killing, as occurs in many neurological diseases, such as Parkinson's disease and Alzheimer's disease. Unfortunately, the toxicity and immunosuppressive effect of cyclosporin A makes it less than ideal for use in acutely ill patients. Thus, an important target for drug discovery by the pharmaceutical industry is the development of blockers of the MPT that lack the adverse toxic and immunosuppressive actions of cyclosporin A. Such pharmaceutical research is likely already under way.

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