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HYPOXIC, ISCHEMIC, AND REPERFUSION INJURY TO LIVER

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HEPATIC OXYGEN METABOLISM (SEE CHAPTER 19 AND WEBSITE CHAPTER W-13)

The liver is a highly aerobic organ whose metabolism and viability depend on the availability of oxygen. Oxygen consumption of the liver is 100 to 150 μmol O_2 per hour per gram of wet weight. The hepatic artery and the portal vein together deliver blood to the liver. These vessels furnish about 25% and 75% of blood flow, respectively, although flow rates vary physiologically, particularly in response to digestive activity. Portal blood is better oxygenated than mixed venous blood but is still less oxygenated than arterial blood. Taking oxygenation into account, the portal vein and hepatic artery each provide roughly half of the oxygen supply to the liver. In most circulations, blood flow is regulated primarily by oxygen demand. In the liver, portal blood flow depends on the activity of the digestive organs and increases during active absorption of nutrients. To a considerable extent, hepatic arterial and venous blood flow are reciprocal so as to maintain constant total blood flow through the liver. Because the liver is an important site of first pass clearance of hormones, stable hepatic blood flow prevents fluctuations in hormone levels that would otherwise occur when hepatic blood flow changes.

Portal and arterial blood thoroughly mix inside the hepatic sinusoid. As sinusoidal blood moves through the hepatic lobules, the liver extracts oxygen, nutrients, bile acids, and hormones (Fig. 18.1). At the same time, synthetic products and metabolic wastes are added to the blood. In this way, sinusoidal blood flow and hepatic metabolism create gradients of oxygen, metabolites, and hormones between periportal and pericentral regions of the liver lobule. Tissue responses to these gradients likely contribute to the development of biochemical differences between hepatocytes in different regions of the hepatic lobule, such as the relative enrichment of ureagenesis, gluconeogenesis, and oxidative metabolism in periportal hepatocytes and xenobiotic and glycolytic metabolism in pericentral hepatocytes (1).

Measurements with microlight guides and miniature oxygen electrodes show directly oxygen gradients within the liver lobule with periportal regions more highly oxygenated than pericentral regions. Although still often cited, earlier measurements claiming that intrahepatic oxygen tension is lower than that of the mixed venous drainage are likely the consequence of the decreased sensitivity of Clark-style oxygen electrodes inside solid tissue. When Clark electrodes are calibrated to oxygen inside solid hepatic tissue, intrahepatic oxygen concentrations are in between that of the inflow and outflow blood vessels (2).

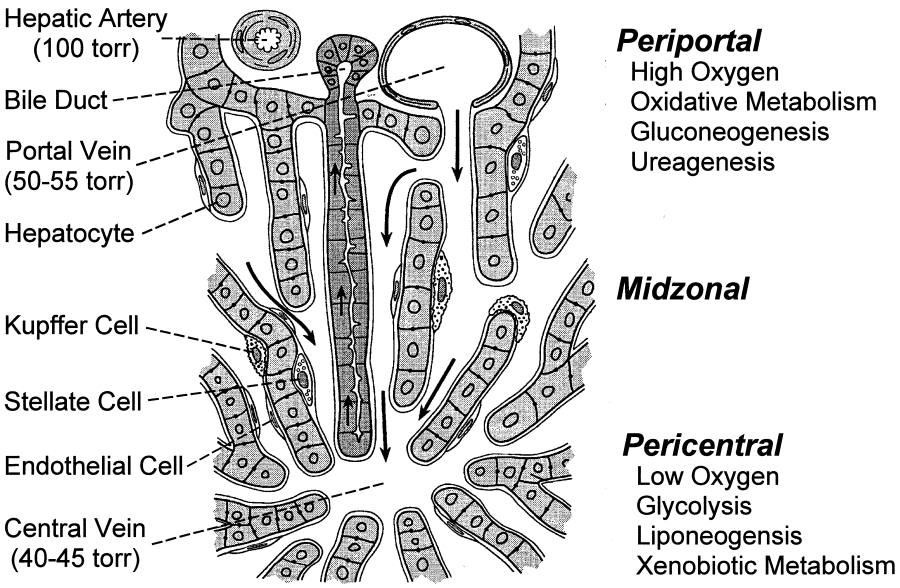


FIGURE 18.1. Oxygen and metabolic gradients within the liver lobule. As blood flows through the liver lobule (*downward arrows*), hepatic tissue extracts oxygen and other blood-borne substances (e.g., hormones, hyaluronic acid, bile acids) while simultaneously adding metabolic wastes and synthetic products. As a consequence, gradients of oxygen and other metabolites form between periportal and pericentral regions. Where blood first enters the lobule, sinusoidal oxygen tension is between that of the hepatic artery and the portal vein. Where blood exits the lobule, oxygen tension is close to that of the central vein (terminal hepatic venule). In low flow and hypoxemic states, pericentral hepatocytes are the first to experience hypoxic stress. Gradients of hepatic enzymes also exist across the liver lobule. Enzymes for oxidative phosphorylation, gluconeogenesis, and ureagenesis are concentrated in periportal regions, whereas enzymes for glycolysis, lipogenesis, and drug metabolism are concentrated in pericentral regions. Bile flow within canaliculi runs countercurrent to blood flow (*upward arrows*).

VULNERABILITY OF LIVER TO HYPOXIC AND ISCHEMIC INJURY

Like the heart and brain, the liver is quite vulnerable to hypoxic injury, but unique features of hepatic vascularization and metabolism afford the liver relative protection against hypoxia. Dual vascularization provides a redundancy of the blood supply, which is the apparent reason why focal ischemic injury secondary to atherosclerosis and related causes is rare in the liver. Livers of well-nourished individuals also contain up to 7% glycogen by weight. This glycogen supports adenosine triphosphate (ATP) generation by anaerobic glycolysis. During anoxia and ischemia, glycolytic ATP formation replaces, in part, ATP lost from oxidative phosphorylation and delays anoxic hepatocellular cell death by hours compared to glycogen-depleted livers.

Even with the protection of a dual blood supply and the anaerobic metabolism of glycogen, hypoxic liver damage is quite common in systemic hypoxemia and cardiogenic, hemorrhagic, and septic shock. Due to the intralobular oxygen gradient, hypoxic injury in low-flow states occurs first in the pericentral region of hepatic lobules (3). Indeed, pericentral and midzonal hepatic necrosis attributable to hypoxic injury is frequently observed at autopsy. If severe enough, pericentral liver hypoxia leads to a syndrome of ischemic hepatitis characterized by a sharp increase in serum transaminase activities in the absence of other causes of hepatic necrosis, such as viral or drug-induced hepatitis (4).

The liver, unlike the heart and brain, has enormous regenerative capacity. Thus, virtually complete restoration of normal liver structure and function can occur after hypoxic injury when normal hepatic perfusion is restored. Repeated cycles of hypoxic injury, however, may lead to chronic liver injury. In alcoholic liver disease, cycles of hypoxic injury are postulated to contribute to hepatic fibrosis and alcoholic cirrhosis (5).

Warm hypoxic liver injury is also of clinical importance in the Budd–Chiari syndrome, veno-occlusive disease, liver surgery, and liver transplantation. The Budd–Chiari syndrome is caused by obstruction of hepatic venous outflow by a thrombus or mass, leading to painful hepatomegaly, microcirculatory stasis, and ascites (6). This outflow obstruction, if untreated by thrombolytic therapy or surgical decompression, leads to progressive hepatic failure requiring liver transplantation. Hepatic veno-occlusive disease, like the Budd–Chiari syndrome, presents clinically as hepatomegaly and ascites most commonly in patients receiving hepatic irradiation and chemotherapy for bone marrow transplantation (7). Veno-occlusive disease is associated with sinusoidal endothelial cell injury and extravasation of red blood cells into the space of Disse. A fibrotic response then leads to obliterative, obstructive lesions of the central veins and smaller hepatic vein branches. Both Budd–Chiari syndrome and veno-occlusive disease cause severe centrilobular congestion and hypoxic hepatocellular necrosis. Ischemia/reperfusion injury is also a concern to the liver surgeon who often needs to occlude branches of the portal vein and hepatic artery temporarily for a blood-free field (Pringle maneuver). Similarly, ischemia/reperfusion injury associated with cold ischemic storage limits liver preservation for transplantation surgery (8).

In animal models, warm ischemia/reperfusion injury to the liver is easily induced by cross-clamping the portal vein and hepatic artery. Clamping the blood supply to specific lobes, such as the median and left lateral lobes, avoids intestinal stasis but still produces ischemia to about 70% of the liver (9). After reflow, hepatic cell death is manifested by release of hepatocellular enzymes (lactate dehydrogenase, transaminases) and uptake of supravital dyes (trypan blue, propidium iodide). Release of enzymes and uptake of normally impermeant dyes signify the breakdown of the plasma membrane permeability barrier, which is the hallmark of onset of necrotic cell death.

CELLULAR CHANGES IN HYPOXIA

One of the earliest hepatocellular changes in hypoxia is the formation of plasma membrane protrusions called blebs (Fig. 18.2) (3,10). Blebs contain cytosol and endoplasmic reticulum but generally exclude larger organelles like mitochondria and lysosomes. Accompanying bleb formation are dilatation of cisternae of endoplasmic reticulum, rounding and moderate swelling of mitochondria, and a 30% to 50% increase of total cellular volume.

These early changes are reversible, and hepatocytes can still recover fully after reoxygenation. Irreversible injury occurs when a plasma membrane bleb literally bursts, causing abrupt 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 (11,12). In hepatocytes, sinusoidal endothelial cells, and other cell types, a metastable state precedes bleb rupture (12). This metastable state begins with mitochondrial permeabilization and lysosomal disruption. Blebs then coalesce and increase in size, low molecular weight anionic fluorophores begin to cross the plasma membrane, and cell swelling accelerates (13). The metastable state culminates in bleb rupture, equilibration of intracellular and extracellular contents, and necrotic cell death.

The cytoprotective amino acid, glycine, inhibits progression into the metastable state after ATP depletion and protects sinusoidal endothelial cells, hepatocytes, and other cell types against the onset of necrotic cell death (14–17). In Madin-Darby canine kidney (MDCK) cells, glycine was reported to block the opening of water-filled pores whose molecular weight exclusion limit increases progressively from about 4,000 d to more than 70,000 d (18). By contrast, in cultured hepatic sinusoidal endothelial cells,

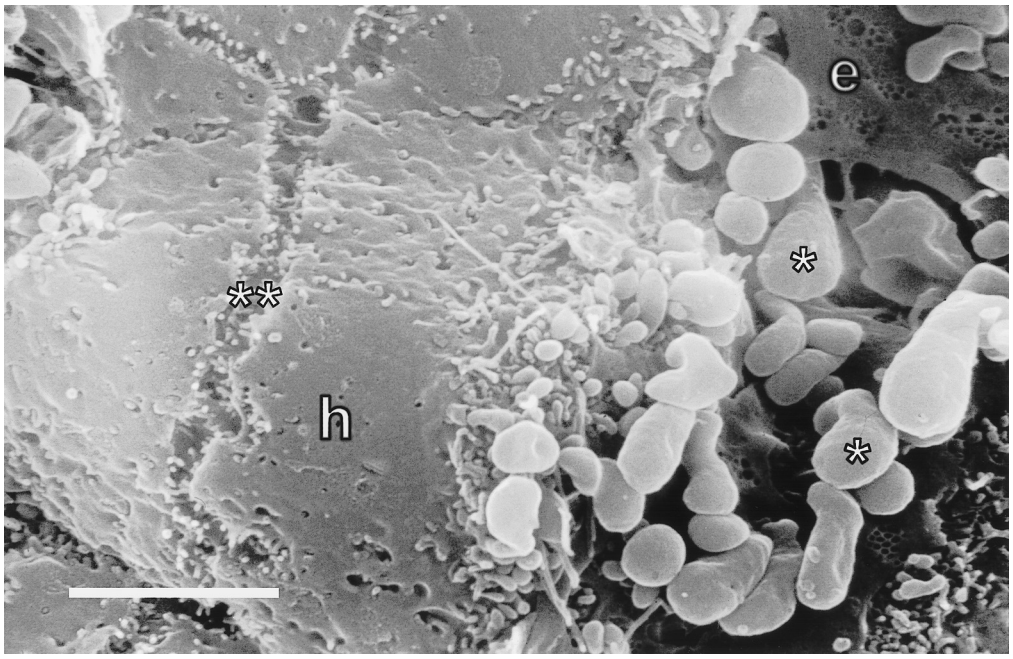


FIGURE 18.2. Scanning electron micrograph of early cell surface bleb formation during hypoxia. Hepatocellular blebs (*single asterisks*) protrude through fenestrations of sinusoidal endothelial cells (*e*) after 15 minutes of low-flow hypoxia in a perfused rat liver. Blebbing occurs on the subsinusoidal surface of the hepatocytes. Intercellular surfaces of the hepatocytes (*h*) are not yet involved, and bile canaliculi (*double asterisks*) are normal. *Bar* is 5 μm. (Adapted from Lemasters JJ, Ji S, Thurman RG. Centrilobular injury following hypoxia in isolated, perfused rat liver. *Science* 1981;213:661–663.)

glycine appeared to inhibit a selective organic anion channel, which is permeable to chloride and polyvalent organic anions up to a molecular size limit of at least 600 d, but not to similarly sized organic cations or larger molecular weight dextrans (17). Opening of the glycine-sensitive organic anion channel leads to the rapid cellular swelling of the metastable state. This swelling likely occurs as Cl^- enters through the glycine-sensitive anion channel and Na^+ enters through monovalent cation channels. The latter channels open earlier in hypoxia (19–21). Colloid osmotic forces drive this swelling, which continues until the plasma membrane bursts. After membrane rupture, cells become permeable to all solutes and further volume growth ceases. Since permeabilization of both mitochondria and lysosomes presage onset of the metastable state (13), a hydrolytic enzyme, such as a protease, or other factor activated by these organelles may be important for opening the glycine gated anion channel.

ADENOSINE TRIPHOSPHATE DEPLETION AND HYPOXIC HEPATOCELLULAR NECROSIS

Failure of aerobic ATP formation by oxidative phosphorylation is the fundamental stress of anoxic and ischemic injury. The importance of ATP depletion in the events leading to necrotic cell death is demonstrated by the ability of glycolytic substrates to rescue hepatocytes and sinusoidal endothelial cells from lethal cell injury (15,22,23). Glycolytic metabolism partially replaces ATP lost after inhibition of mitochondrial oxidative phosphorylation, and ATP at only 15% to 20% of normal levels is sufficient to prevent onset of necrotic cell death. Glucose, the major glycolytic substrate for most cell types, prevents hypoxic killing of sinusoidal endothelial cells, but glucose does not protect hepatocytes against anoxic injury. Glucose is ineffective in hepatocytes because hepatocytes lack hexokinase. Hexokinase catalyzes the first reaction of glycolysis in most cells and has a high maximum velocity (V_{max}) and a low Michaelis' constant (K_m) for glucose. Hepatocytes have instead glucokinase with a high K_m for glucose and relatively low V_{max}. Glucokinase is metabolically appropriate for hepatocytes, since the liver has the important function of maintaining blood glucose to a concentration of about 5 mM. Even under conditions of anoxia, hepatic consumption of glucose is very low, because rapid hepatic glucose utilization would otherwise lead to systemic hypoglycemia. Instead, fructose protects hepatocytes against hypoxic injury, because hepatocytes contain a highly active fructokinase that feeds fructose into the glycolytic pathway.

In hepatocytes, endogenous glycogen is also an excellent substrate for anaerobic glycolysis. For this reason, hepatocytes of glycogen-rich livers from fed rats are much more resistant to anoxic killing than hepatocytes of glycogen-depleted livers of fasted rats (24). Fructose acts as an alternate glycolytic substrate and prevents anoxic hepatocellular

damage in glycogen-depleted livers. Fructose also prevents hepatocellular killing by several toxic chemicals, which implies that mitochondria are important targets of toxic cell killing (25,26). Consistent with these observations in experimental animals, glycogen depletion after fasting predisposes human subjects to acetaminophen-induced liver damage (27).

In aerobic livers, high fructose causes a decrease of ATP and inorganic phosphate (P_i) because of ATP consumed in the fructokinase reaction and the consequent accumulation of sugar phosphate metabolic intermediates. This decline of ATP is often assumed to represent fructose toxicity despite the fact that glucose causes a similar decline of ATP in hexokinase-containing cells. Actually, fructose-treated livers maintain their ATP/adenosine diphosphate (ADP)•P_i ratios, because fructose-induced decreases of ATP are offset by decreases of P_i. The ATP/ADP•P_i ratio is proportional to the free energy of hydrolysis of ATP or phosphorylation potential (ΔG_p). ΔG_p rather than ATP concentration, ATP/ADP ratio, or energy charge is the relevant thermodynamic variable reflecting the energy available from ATP. Furthermore, during anoxia when ATP falls to virtually immeasurable levels, fructose metabolism actually increases ATP substantially. During anoxic and toxic stress, this ATP generation prevents hepatocellular killing (28). Thus, fructose-induced changes of ATP reflect the normal hepatic metabolism of fructose rather than fructose toxicity.

In anoxia, mitochondrial respiration and hence oxidative phosphorylation become fully inhibited. Respiratory inhibitors, such as cyanide and antimycin A, mimic many of the features of hypoxic injury in an experimental model sometimes called "chemical hypoxia" (11). A more severe form of mitochondrial metabolic disruption is uncoupling, which occurs when the mitochondrial inner membrane becomes permeable to hydrogen ions. Uncoupling activates the mitochondrial F_1F_0 adenosine triphosphatase (ATPase). This mitochondrial ATPase normally acts in the reverse direction as the ATP synthase of oxidative phosphorylation. Activation of mitochondrial ATPase by uncoupling causes futile hydrolysis of ATP. As a consequence, glycolytic ATP generation can no longer protect against cell killing. Inhibition of the mitochondrial ATPase with oligomycin prevents mitochondrial hydrolysis of glycolytic ATP after uncoupling and restores the cytoprotection of glycolysis. In the absence of glycolytic substrate, oligomycin actually induces cell killing because it inhibits ATP formation by oxidative phosphorylation. However, in the presence of a glycolytic substrate such as fructose, oligomycin prevents cell killing induced by mitochondrial uncoupling (25,28).

These experimental findings illustrate that mitochondria undergo a progression of injurious changes in response to external stresses (Fig. 18.3). Simple inhibition of mitochondrial respiration with agents such as anoxia or cyanide blocks oxidative phosphorylation. In the absence of glycolysis, respiratory inhibition leads to cellular ATP depletion

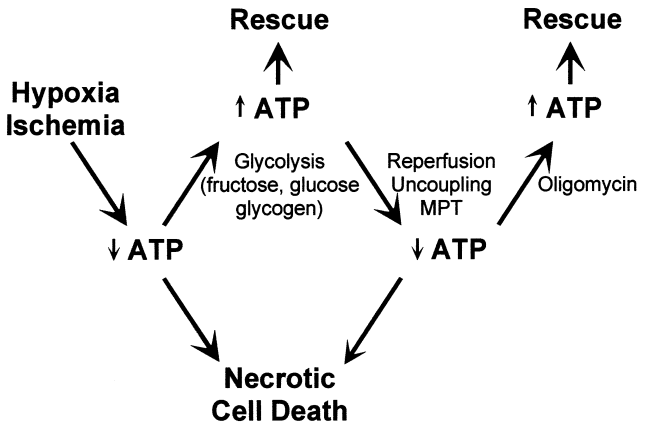


FIGURE 18.3. Progression of mitochondrial injury during hypoxia/ischemia and reperfusion. Oxygen deprivation during ischemia inhibits mitochondrial oxidative phosphorylation, which leads to adenosine triphosphate (*ATP*) depletion and necrotic cell death. Glycolysis restores ATP and prevents anoxic cell killing. Reperfusion induces onset of the mitochondrial permeability transition (*MPT*) and mitochondrial uncoupling, which activates the mitochondrial F_1F_0 adenosine triphosphatase (ATPase). This ATPase futilely hydrolyzes ATP made available by glycolysis to overcome the protective effect of glycolytic substrates such as fructose, glucose, and glycogen. Oligomycin inhibits the mitochondrial ATPase, restores glycolytic ATP levels and rescues cells from necrotic cell death. (Adapted from Nieminen AL, Saylor AK, Herman B, et al. ATP depletion rather than mitochondrial depolarization mediates hepatocyte killing after metabolic inhibition. *Am J Physiol* 1994;267:C67–C74.)

and ultimately necrotic cell death. Glycolytic substrates such as fructose in hepatocytes and glucose in sinusoidal endothelial cells partially restore ATP levels and rescue the cells from necrotic killing. However, when mitochondria become uncoupled, then the mitochondrial synthase works in reverse to hydrolyze ATP made available by glycolysis. As a consequence, ATP levels fall profoundly even in the presence of glycolytic substrates, and lethal cell injury ensues. Under these conditions, oligomycin prevents cell killing due to mitochondrial uncoupling by blocking futile uncoupler-induced hydrolysis of glycolytic ATP.

Several toxicants produce cytotoxicity by mitochondrial uncoupling, as shown by cytoprotection with fructose plus oligomycin. These toxicants include 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*-butylhydroperoxide (25,29). In anoxia/ischemia, free fatty acids accumulate due to activation of phospholipases and inhibition of fatty acid acylation. Free fatty acids are weak mitochondrial uncouplers and may contribute to anoxic and ischemic injury.

PROTECTION BY ACIDOTIC PH AGAINST HYPOXIC KILLING OF LIVER CELLS

Anoxia and *hypoxia* are terms indicating oxygen deprivation that are often used somewhat interchangeably. Anoxia refers

to an absolute absence of oxygen, whereas hypoxia refers to relative but not necessarily absolute oxygen deprivation. Ischemia means the loss of blood supply, which can also be relative or absolute. Tissue injury and stress in ischemia begin to occur as tissue oxygen levels approach very low levels. Ischemia produces other tissue changes, particularly a rapid decrease of pH, which can fall by a unit or more (30). This naturally occurring acidosis greatly delays onset of necrotic cell death in hepatocytes and many other cells despite exhaustion of cellular ATP supplies (31–33). Intracellular acidification mediates the protection of acidotic pH. Although anaerobic metabolism contributes to the decline of pH in ischemia, hydrogen ion generation from hydrolysis of high-energy phosphates such as ATP and the release of hydrogen ions from acidic organelles also contribute to cytosolic acidification (32,34). Intracellular acidosis may suppress one or more intracellular enzymes activated by hypoxic stress, such as phospholipase A. ATP depletion activates phospholipase A, phospholipase inhibitors delay hypoxic cell killing, and acidic pH inhibits phospholipase A activity stimulated by hypoxic stress. Phospholipases may in turn activate proteases that further promote cell injury (35).

THE PH PARADOX IN EARLY ISCHEMIA/REPERFUSION INJURY

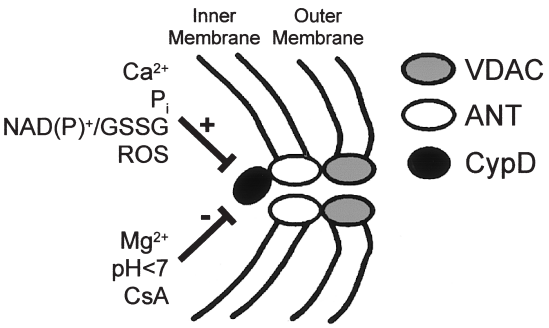
Although the naturally occurring acidosis of ischemia prevents onset of anoxic cell death, reperfusion after ischemia can paradoxically worsen cell injury and precipitate tissue necrosis within minutes. In experimental models, anoxia at acidotic pH followed by reoxygenation at pH 7.4 simulates oxygen deprivation and acidosis during ischemia and recovery of oxygen and pH after reperfusion. Reperfusion under these conditions leads to loss of cell viability and release of intracellular enzymes such as lactate dehydrogenase (31, 36–40). Restoration of normal pH after reperfusion rather than reoxygenation causes this injury, since reoxygenation at low pH prevents cell killing virtually entirely, whereas return to normal pH without reoxygenation produces the same cell killing as return to normal pH with reoxygenation. This paradoxical injury after recovery of normal pH is called the pH paradox.

Intracellular pH mediates cell injury in the pH paradox. If recovery of intracellular pH is accelerated during reperfusion with an ionophore such as monensin, cell killing occurs more quickly. Conversely, inhibition of the rise of intracellular pH after reperfusion by Na^+/H^+ exchange blockade with dimethylamiloride (in cardiac myocytes) or Na^+-free medium (in hepatocytes) prevents reperfusion-induced necrotic cell killing almost completely. pH-dependent cell killing is independent of extracellular and cytosolic Ca^{2+} and Na^+ and is not linked to pH-dependent secondary changes of cytosolic Na^+ and Ca^{2+} (32,37–39, 41,42).

THE MITOCHONDRIAL PERMEABILITY TRANSITION IN REPERFUSION INJURY

Recent studies in a variety of tissues implicate onset of a phenomenon called the mitochondrial permeability transition in lethal cell injury associated with anoxia, reperfusion, and oxidative stress (reviewed in ref. 43). In the mitochondrial permeability transition, mitochondria become freely permeable to solutes of molecular weight less than about 1500 d (Fig. 18.4) (44). Ca^{2+}, P_i, reactive oxygen species, and numerous oxidant chemicals induce the mitochondrial permeability transition, whereas Mg^{2+}, low pH, and the immunosuppressant drug cyclosporin A block the mitochondrial permeability transition. However, inhibition of the mitochondrial permeability transition by cyclosporin A is unrelated to its immunosuppressive action. At onset of the mitochondrial permeability transition, mitochondria depolarize and undergo large-amplitude swelling. As a consequence, oxidative phosphorylation becomes uncoupled. Patch clamping identifies a highly conductive permeability transition (PT) pore in the mitochondrial inner membrane whose opening causes the mitochondrial permeability transition (45).

The PT pore is likely composed, at least in part, of the adenine nucleotide translocator (ANT) protein in the mitochondrial inner membrane together with cyclophilin D (a cyclosporin A–binding protein) in the mitochondrial matrix, and porin in the outer membrane (Fig. 18.4) (46,47). Translocation of the proapoptotic protein Bax to



Mitochondrial Permeability Transition

- From opening of a high conductance permeability transition pore conducting solutes up to 1,500 Da.
- Pore formed by adenine nucleotide translocator (ANT), cyclophilin D (CypD) and the voltage dependent anion channel (VDAC).
- Causes mitochondrial depolarization, uncoupling and swelling.
- Promoted by Ca^{2+}, inorganic phosphate (Pi), NAD(P)H/GSH oxidation and reactive oxygen species (ROS).
- Inhibited by Mg^{2+}, low pH, and cyclosporin A (CsA).

FIGURE 18.4. Properties and postulated molecular composition of the permeability transition pore.

the mitochondrial surface also promotes opening of the PT pore (48). This combination of proteins implies that the PT pore spans the inner and outer membrane, presumably at contact sites between the mitochondrial inner and outer membranes. However, our understanding of the exact molecular structure of the PT pore remains incomplete.

pH below 7.0 inhibits the PT pore, and involvement of the mitochondrial permeability transition in pH-dependent reperfusion injury is supported by the observation that cyclosporin A prevents pH-dependent killing of cultured rat hepatocytes after simulated ischemia/reperfusion, even when added only during the reperfusion phase (41). Cyclosporin A also protects heart and brain against anoxia/reoxygenation injury (49,50). Laser scanning confocal microscopy confirms directly a role of the mitochondrial permeability transition in pH-dependent reperfusion injury. When hepatocytes subjected to simulated ischemia are reoxygenated and returned from acidotic pH to normal pH, mitochondria initially begin to repolarize (Fig. 18.5). However, after several minutes the nonspecific permeability of the mitochondrial inner membrane abruptly increases as intracellular pH rises to about 7.0. This is shown by the movement of a normally impermeant fluorophore, calcein, from the cytosol into the mitochondria. Simultaneously, the mitochondria depolarize. Loss of cell viability then occurs after several more minutes (Fig. 18.5). If hepatocytes are reoxygenated at acidic pH or at normal pH in the presence of cyclosporin A, then mitochondrial permeabilization does not occur, and recovery of mitochondrial membrane potential is sustained. Moreover, cell death does not occur. Instead, surface blebs disappear and cellular swelling reverses, indicating restoration of ATP-dependent cellular processes. These findings support the conclusion that the mitochondrial permeability transition is a major causative mechanism in the pathogenesis of reperfusion injury to hepatocytes (41). The mitochondrial permeability transition also plays a causative role in hepatocellular injury due to oxidative stress, various toxicants including Reye's syndrome-related drugs, and cellular calcium overload (43).

pH-dependent reperfusion injury is linked directly to the recovery of intracellular pH from the intracellular acidosis of ischemia. Monensin, a Na^+,H^+-exchanging ionophore that accelerates the recovery of intracellular pH after reperfusion, also accelerates pH-dependent reperfusion injury. Conversely, blockade of plasma membrane Na^+/H^+ exchange by dimethylamiloride (in cardiac myocytes) or a Na^+-free medium (in hepatocytes) delays recovery of intracellular pH after reperfusion and prevents reperfusion-induced loss of cell viability almost completely (38,39,41,42). pH-dependent cell killing occurs independently of levels of cytosolic and extracellular free Ca^{2+}, although a specific increase of mitochondrial free Ca^{2+} precedes onset of mitochondrial permeabilization in both oxidative stress and pH-dependent reperfusion injury (51,52). Intracellular Na^+ increases relatively early during ATP depletion (53). The plasma mem-

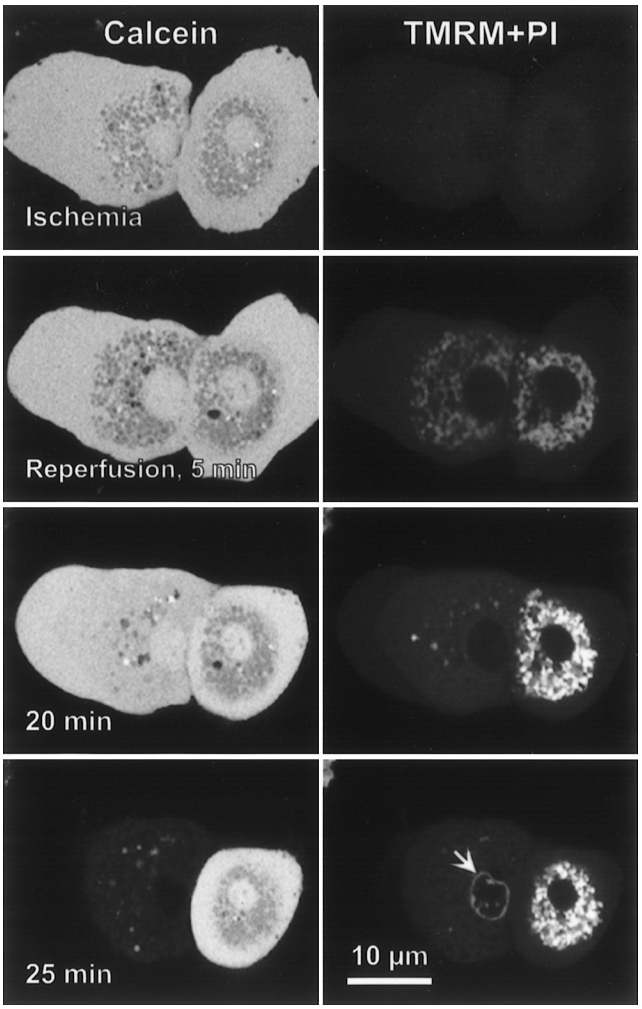


FIGURE 18.5. Induction of the mitochondrial permeability transition by ischemia and reperfusion. Cultured rat hepatocytes were co-loaded with green-fluorescing calcein (**left panels**) and red-fluorescing tetramethylrhodamine methylester (*TMRM*, **right panels**) and subjected to anoxia at pH 6.2, conditions that simulate ischemia. At the end of 4 hours of anoxia, mitochondria were small dark round voids in confocal images of calcein fluorescence, which indicated that the mitochondrial inner membrane remained impermeable to this 623-Da polyanionic fluorophore in the cytosol. At this time, mitochondria were depolarized and did not accumulate TMRM, a membrane potential indicating cationic fluorophore. After rexoxygenation at pH 7.4 to simulate reperfusion, TMRM began to enter the mitochondria of both hepatocytes in the field within 5 minutes. After 20 minutes, one of the hepatocytes then lost TMRM labeling, which indicated mitochondrial depolarization, and its mitochondria filled with calcein, which indicated onset of the mitochondrial permeability transition. The hepatocyte then lost viability after 25 minutes, as indicated by release of cytosolic calcein and nuclear uptake of propidium iodide (PI, *arrow*) included in the incubation medium. The other hepatocyte in the field did not lose viability, and its mitochondria continued to accumulate TMRM and exclude calcein. (Adapted from Qian T, Nieminen AL, Herman B, et al. Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am J Physiol* 1997;273:C1783–C1792.)

brane Na^+/H^+ exchanger mediates, in part, this increase. However, prevention of intracellular Na^+ loading by acidotic pH does not account for cytoprotection by acidotic pH, because acidotic pH protects against cell killing even when intracellular Na^+ and extracellular Na^+ are equilibrated with monensin (32).

REACTIVE OXYGEN SPECIES AND REPERFUSION INJURY

Reoxygenation of hypoxic liver also promotes the formation of reactive oxygen species, including hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$). Sources of reactive oxygen species include xanthine oxidase utilizing xanthine and hypoxanthine generated after ATP degradation, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in Kupffer cells activated by ischemic stress, and the respiratory chain of mitochondria. In the presence of transition metal ions, such as free iron and copper, H_2O_2 and $O_2^{\bullet-}$ react to form the highly reactive and toxic hydroxyl radical (OH•) by the Fenton reaction (Fig. 18.6). In addition, iron catalyzes a lipid peroxidation chain reaction sustained by lipid alkyl and peroxyl radicals. The iron chelator desferal blocks these iron-catalyzed reactions. Superoxide also reacts nonenzymatically with nitric oxide (NO·) to form peroxynitrite ($OONO^-$). Peroxynitrite causes nitrosylation of tyrosyl residues in proteins and also decomposes to a hydroxyl radical-like species. Increasingly, peroxynitrite is recognized as an important toxic intermediate in oxidative tissue injury (54).

Although pH-dependent reperfusion injury occurs in the absence of oxygen and therefore of reactive oxygen species formation, oxidative stress nonetheless also promotes onset of the mitochondrial permeability transition, as shown in hepatocytes treated with *tert*-butylhydroperoxide, a short-chain analogue of the lipid hydroperoxides formed during oxidative stress and ischemia/reperfusion (55). This oxidant chemical initiates a chain of events that culminates in the mitochondrial permeability transition and necrotic cell death. The earliest effect of *tert*-butylhydroperoxide is oxidation of mitochondrial pyridine nucleotides [reduced nicotinamide adenine dinucleotide (NADH) and NADPH] and glutathione, which is followed by an increase of intramitochondrial free Ca^{2+}. Increased mitochondrial Ca^{2+} then stimulates mitochondrial reactive oxygen species formation, which leads to PT pore opening, mitochondrial depolarization, ATP depletion, and cell death (51,56).

In low-flow states, pericentral regions of the liver lobule become anoxic, whereas periportal areas remain normoxic.

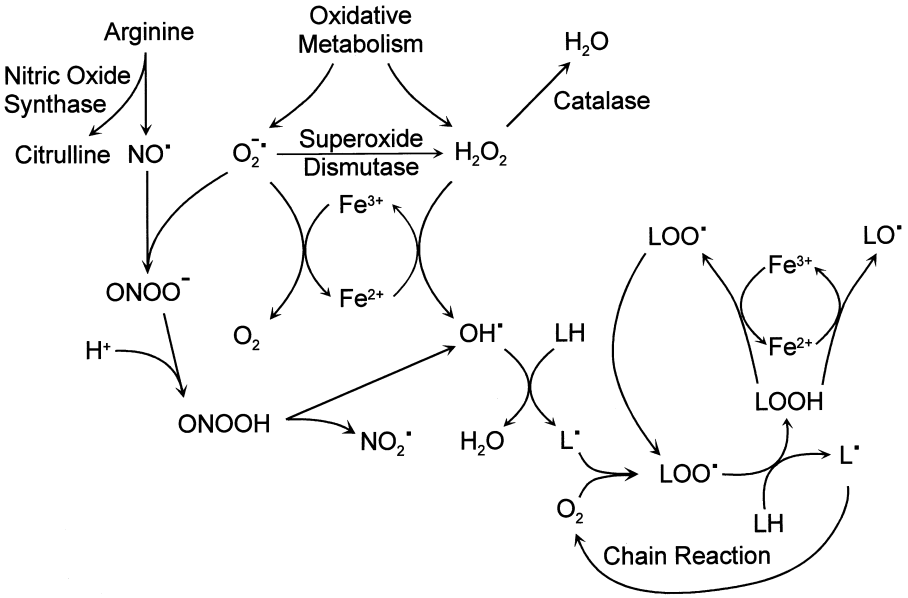


FIGURE 18.6. Iron-catalyzed free radical generation. Oxidative metabolism after reperfusion leads to formation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Superoxide is detoxified to hydrogen peroxide by superoxide dismutase, and hydrogen peroxide is converted to water by catalase. Iron and other transition metals, including copper, catalyze hydroxyl radical (OH•) formation by the Haber Weiss reaction. Superoxide reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), which reacts with hydrogen peroxide to form the highly reactive hydroxyl radical. Hydroxyl radicals react with lipids to form alkyl radicals (L•) that initiate an oxygen-dependent chain reaction generating peroxyl radicals (LOO•) and lipid peroxides (LOOH). Lipid peroxides react with free iron to generate alkoxyl radicals (LO•) and more peroxyl radicals. Nitric oxide synthase catalyzes nitric oxide (NO•) formation from arginine. Nitric oxide reacts nonenzymatically with superoxide to form the unstable peroxynitrite anion ($ONOO^-$), which protonates and decomposes to nitrogen dioxide and hydroxyl radical. These toxic radicals also attack proteins and nucleic acids.

The border between normoxic and anoxic tissue is sharp, as reflected by an increase of reduced pyridine nucleotides [nicotinamide adenine dinucleotide (NAD) plus nicotinamide adenine dinucleotide phosphate (NADP)] whose reoxidation is prevented by anoxia (3). Such midzonal border regions are the sites of formation of toxic reactive oxygen species (57). At this border region, the coexistence of hypoxic stress and small amounts of oxygen promotes an accelerated midzonal injury that is blocked by antioxidants. A midzonal pattern of hepatic necrosis is also frequently observed at autopsy after liver hypoperfusion (58).

APOPTOSIS

Apoptosis is another mode of cell death that leads to cell deletion without the inflammation, scarring, and release of cellular contents that characterize necrotic cell death (Table 18.1) (see Chapter 19 and website chapter W-13). In apoptosis, individual cells shrink and separate from their neighbors. Other characteristic changes of apoptosis include alterations of plasma membrane lipids, condensation of chromatin, internucleosomal DNA degradation, and shedding of membrane-bound cytoplasmic fragments containing ultrastructurally intact organelles and chromatin. Adjacent cells and macrophages take up these apoptotic bodies. In liver pathology, they are Councilman bodies, a characteristic feature of hepatocellular apoptotic cell death (59). Specific physiologic death signals, such as tumor necrosis factor-α (TNF-α) and Fas ligand, trigger apoptosis through a cascade of cysteine-aspartate proteases called caspases.

Apoptosis is also a late sequela of ischemia/reperfusion injury in liver and other tissues and occurs in cells that survive acute onset of necrotic cell death (60,61). Mitochondrial changes, specifically the mitochondrial permeability transition, induced by ischemia reperfusion may play a role in this apoptosis. When purified nuclei and isolated mitochondria are combined in a cell-free system, onset of the mitochondrial permeability transition induces the release of soluble factors from mitochondria that activate caspases and initiate apoptotic nuclear changes (62). These factors include the loosely bound respiratory protein, cytochrome *c*, and apoptosis-inducing factor (AIF), which reside in the space between the mitochondrial inner and outer membranes (63,64). Release of cytochrome *c* occurs when large-amplitude mitochondrial swelling following the mitochondrial permeability transition causes rupture of the outer membrane. Other mechanisms, including the formation of specific cytochrome *c* release channels in the outer membrane by proapoptotic Bcl2 family members such as Bax, are also proposed to explain the release of cytochrome *c* and other proapoptotic mitochondrial factors during apoptosis (65,66).

After release from mitochondria, cytochrome *c* binds to apoptosis-inducing factor-1 (APAF-1) (67). APAF-1 also binds deoxyATP (or dATP) and pro-caspase 9 to form a complex that yields a proteolytically activated caspase 9. Caspase 9, in turn, proteolytically activates pro-caspase 3 to caspase 3, which then initiates the final execution stages of apoptosis, including cell shrinkage, surface blebbing, internucleosomal DNA hydrolysis, chromatin margination, and nuclear lobulation. Other caspases, such as caspase 8, act upstream of mitochondria. For example, binding of TNF-α and Fas ligand to their receptors leads to pro-caspase 8 activation (Fig. 18.7). Caspase 8 then cleaves Bid, another member of the proto-oncogene Bcl2 family of proteins (68), to a truncated form that translocates to mitochondria and induces cytochrome *c* release. Other pro- and anti-apoptotic members of the Bcl2 family also bind to mitochondria to promote or block, respectively, mitochondrial permeabilization and cytochrome *c* release. In particular, the protein Bcl2 blocks cytochrome *c* release and prevents apoptotic signaling through mitochondria (69).

The specific role of the mitochondrial permeability transition in apoptosis is the subject of ongoing controversy. Some studies conclude that release of cytochrome *c* during apoptosis occurs without mitochondrial depolarization or onset of the mitochondrial permeability transition. In hepa-

TABLE 18.1. FEATURES OF NECROTIC AND APOPTOTIC CELL DEATH

Necrosis	Apoptosis
Accidental cell death	Controlled cell deletion
Contiguous regions of cells	Individual cells separating from their neighbors
Cell swelling	Cell shrinkage
Large plasma membrane blebs without organelles	Zeiotic blebs containing large organelles
Small chromatin aggregates	Condensation of chromatin and nuclear lobulation
Random DNA degradation (smear on gel)	Internucleosomal DNA degradation (ladder on gel)
Cell lysis and release of intracellular contents	Fragmentation into apoptotic bodies
Marked inflammation and scarring	Absence of inflammation and scarring
Mitochondrial swelling and dysfunction	Mitochondrial permeabilization
Phospholipase and protease activation	Caspase activation
ATP depletion and metabolic disruption	ATP and protein synthesis sustained
Cell death precipitated by plasma membrane rupture	Intact plasma membrane

ATP, adenosine triphosphate.

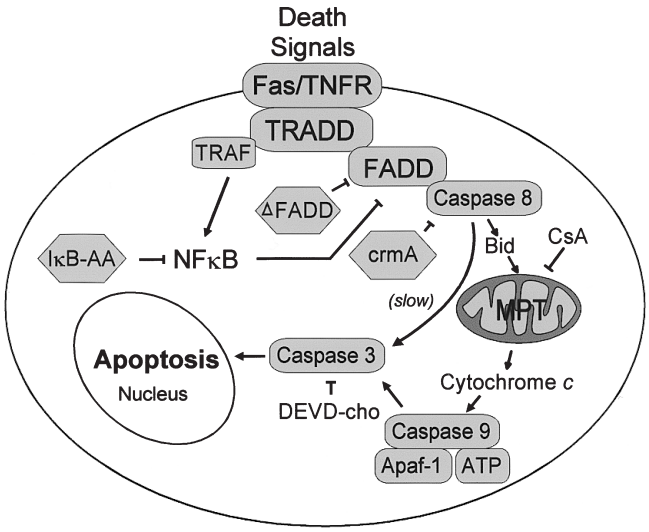


FIGURE 18.7. Scheme of molecular events in death receptor-induced apoptosis. Binding of death signals, such as Fas ligand and tumor necrosis factor-α (TNF-α), to their receptors, Fas or a TNR-α receptor (*TNFR*), activates caspase 8 via the adapter proteins, TNFR-associated death domain (*TRADD*), and Fas-associated death domain (*FADD*). Caspase 8 cleaves the proapoptotic Bcl2 family member, Bid, which then translocates to mitochondria. Subsequently, the mitochondrial permeability transition (*MPT*) occurs, leading to release of cytochrome *c*. Cyclosporin A (CsA) blocks mitochondrial permeabilization. Cytochrome *c* binds to apoptosis-inducing factor-1 (Apaf-1) and ATP. This complex activates caspase 9, which in turn proteolytically activates caspase 3. Caspase 3 then initiates the major biochemical and morphologic manifestation of apoptosis. Signaling through another adapter protein, TNF receptor-associated factor (*TRAF*), activates the nuclear transcription factor NFκB. NFκB induces antiapoptotic gene expression acting upstream of mitochondria. Adenoviral expression of an IκB superrepressor, IκB-AA, inhibits the activation of NFκB and is permissive for TNF-α–induced apoptosis to hepatocytes. Adenoviral expression of CrmA, a serpin family protease inhibitor from cowpox virus, inhibits caspase 8 and blocks the mitochondrial permeability transition after TNF-α. Expression of ΔFADD, a truncated dominant-negative FADD, also blocks upstream signaling. Inhibition of downstream caspase 3 with DEVD-cho prevents apoptosis but not mitochondrial permeabilization. Caspase 8 can also activate caspase 3 directly bypassing mitochondria, but this reaction is slow in hepatocytes.

tocytes, however, onset of the mitochondrial permeability transition can be directly visualized after TNF-α treatment and Fas ligation from movement of the normally impermeant green fluorophore calcein into mitochondria from the cytosol (70,71). This mitochondrial permeability transition precedes cytochrome *c* release, caspase 3 activation, and apoptotic cell death. Cyclosporin A blocks mitochondrial permeabilization induced by TNF-α and Fas ligation in hepatocytes and inhibits cytochrome *c* release, caspase 3 activation, and apoptosis. Another slower proapoptotic signaling pathway coexists with the mitochondrial pathway. Thus, in the presence of cyclosporin A, apoptosis in hepatocytes may be delayed rather than prevented, in which case apoptosis occurs without mitochondrial permeabilization, depolarization and cytochrome *c* release (Fig. 18.7). Apoptotic signaling that bypasses mitochondria is the so-called type 1 pathway and may involve exaggerated activation of caspase 8, whereas apoptotic signaling requiring mitochondrial changes is the type 2 pathway (72).

ADENOSINE TRIPHOSPHATE SWITCH BETWEEN NECROTIC AND APOPTOTIC CELL DEATH

The effect of the mitochondrial permeability transition on ATP is an important factor determining whether apoptosis or necrosis follows onset of the mitochondrial permeability transition. Apoptosis is an ATP-requiring process (73,74), and caspase 9 activation by the cytochrome *c*/APAF-1 complex requires ATP or dATP (63,67). Necrotic cell death, by contrast, is the consequence of ATP depletion (28). Thus, when the mitochondrial permeability transition develops slowly and heterogeneously within a hepatocyte without fully depleting ATP levels, apoptosis can develop, but when the mitochondrial permeability transition is so rapid and extensive that cellular ATP virtually disappears, then necrotic cell death occurs (Fig. 18.8). A not infrequent event in cells undergoing apoptosis is so-called secondary necrosis, in which necrotic cell killing with breakdown of

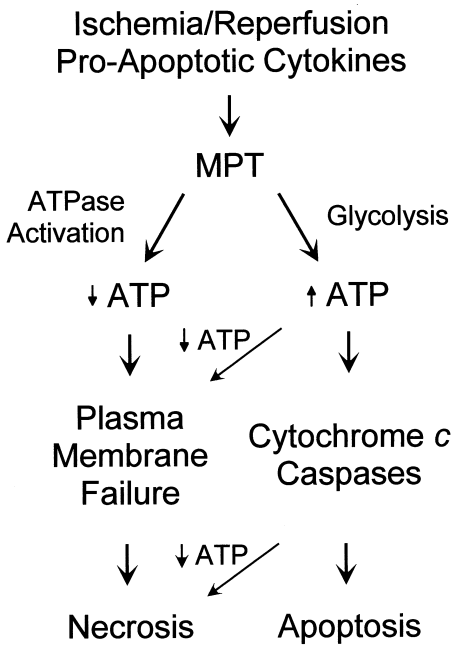


FIGURE 18.8. Role of adenosine triphosphate (*ATP*) in initiating necrosis or apoptosis after the mitochondrial permeability transition. Ischemia/reperfusion and cytokine death signals promote onset of the mitochondrial permeability transition (*MPT*). When mitochondrial permeabilization occurs abruptly, activation of mitochondrial ATPases causes ATP depletion, which leads to plasma membrane failure and necrotic cell death. If mitochondrial permeabilization progresses slowly, glycolysis can maintain ATP levels. In the presence of ATP, cytochrome *c* release from mitochondria activates cytosolic caspases, causing apoptotic rather than necrotic cell death. At any time, ATP depletion may supervene to induce secondary necrosis.

the plasma membrane permeability barrier occurs as apoptosis is progressing. Secondary necrosis may develop from ATP depletion due to mitochondrial failure (Fig. 18.8).

After ischemia/reperfusion, viral infection, and exposure to toxic chemicals, apoptotic and necrotic features often coexist. For example, massive apoptosis in mouse livers after injection of anti-Fas antibody leads to fulminant hepatic failure, disruption of liver architecture, enzyme release, and liver inflammation, features usually associated with necrosis (75). Moreover, pharmacologic inhibition of apoptosis prevents this liver inflammation (59). Not surprisingly, controversies have developed as to whether cell killing in a particular setting is apoptosis or necrosis (76,77), because conventional distinctions between apoptotic and necrotic cell death often do not hold in pathologic situations. Recently, the term *necrapoptosis* was introduced 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) depending on other modifying factors (78). In necrapoptosis, pure apoptosis and pure necrosis are extremes in a continuous spectrum, and the more typical pathophysiologic response is a mixture of features associated with apoptotic and necrotic cell death.

ROLE OF KUPFFER CELLS IN ISCHEMIA/REPERFUSION INJURY

Kupffer cells are the resident macrophages of the liver, which reside in the lining of the hepatic sinusoids directly facing the blood. Ischemia/reperfusion activates Kupffer cells, and this activation also contributes to anoxic, ischemic, and reperfusion injury to liver (see Chapters 19, 30, and 31 and website chapters W-13 and W-26). Activated Kupffer cells release cytokines, reactive oxygen species, and other factors that aggravate ischemia/reperfusion injury and promote postischemic neutrophil infiltration and oxidative stress (reviewed in ref. 79). Even prior to reoxygenation, Kupffer cells enhance anoxic killing of hepatocytes in perfused rat livers (80). Kupffer cells also mediate delayed responses occurring up to 24 hours after reperfusion. An intermediate response occurs up to 6 hours after reperfusion and involves release of cytokines, chemokines, reactive oxygen species, and other mediators by Kupffer cells that act to expand the reperfusion injury. A later injury up to 24 hours following reperfusion results from the hepatic infiltration and activation of neutrophils in response to chemoattractants produced by Kupffer cells.

The cytokines produced by activated Kupffer cells include TNF-α and interleukin-1 and -6 (IL-1 and IL-6) (81) (Chapter 40). TNF-α enhances oxidative stress-induced injury and induces apoptosis in hepatocytes, provided that protein synthesis or NFκB-mediated gene expression is suppressed (70,82). Liver-derived TNF-α also induces release of chemokines from the liver, including epithelial neutrophil activating protein (ENA-78), cytokine-induced neutrophil chemoattractant (CINC), macrophage inflammatory protein (MIP-2), monocyte chemoattractant (MCP-1), and others (83–85). These chemokines are strong chemotactic agents for neutrophils. MIP-2 and CINC also increase integrin expression on neutrophils, which further promotes neutrophil margination into the hepatic microvasculature (86).

Like TNF-α, IL-1 increases within minutes of ischemia/reperfusion injury *in vivo* and promotes reactive oxygen species formation (81,87). By contrast, IL-6 release is delayed, and IL-6 treatment protects against warm ischemia/reperfusion injury in rats (88). Kupffer cells are a major source of reactive oxygen species during the intermediate phase of ischemia/reperfusion injury, whereas neutrophils are the major reactive oxygen species source during the late phase of injury (89–92). Oxidative stress is demonstrated by decreased glutathione levels and elevated oxidized glutathione (GSSG) levels (93). Furthermore, treatment with antioxidants, such as superoxide dismutase, *N*-acetylcysteine, desferal, and allopurinol can decrease hepatic ischemia/reperfusion injury and improve survival (57, 94–98). Lipid peroxidation is modest after hepatic ischemia/reperfusion compared to that induced by strong oxidant chemicals. This suggests that reactive oxygen species do not cause cytotoxicity directly, but act as signaling molecules to upregulate nuclear transcription factors like NFκB and subsequent release of TNF-α and IL-1 (99).

Complement (C3a and C5a) generation occurs after ischemia/reperfusion and promotes Kupffer cell oxidant stress (100,101). Platelet activating factor (PAF) also promotes warm reperfusion injury since PAF receptor blockade decreases hepatic damage and improves survival after liver ischemia/reperfusion (102,103). The source of PAF is undefined, but platelets, Kupffer cells, endothelial cells, and neutrophils all can release PAF. PAF increases vascular permeability, and stimulates neutrophils, macrophages, and monocytes to release superoxide, IL-6, IL-8, and TNF-α (102).

MICROCIRCULATORY CHANGES AND THE PROTECTIVE ACTION OF NITRIC OXIDE

Ischemia/reperfusion disturbs the hepatic microcirculation and causes focal narrowing of the sinusoids produced, in part, by obstruction of flow by swollen Kupffer cells (104,105). Endothelin-1 (ET-1), a potent vasoconstrictor released by endothelial cells and stellate cells, also contributes to microcirculatory disturbances (106,107). In addition, neutrophil promote ET-1 formation by releasing proteases that cleave inactive "big ET" to its active form (107,108).

Nitric oxide (NO) generation appears to be a protective response of liver tissue to ischemia/reperfusion. Nitric oxide

is a vasodilator that improves hepatic oxygenation and sinusoidal microcirculation. Nitric oxide can be rapidly formed by endothelial cells following shear stress and during hepatic hypoperfusion (109). Inhibition of NO synthase decreases hepatic oxygenation and sinusoidal blood flow, increases adhesion molecule expression, and increases hepatocellular damage after ischemia/reperfusion, whereas L-arginine, an NO precursor, improves blood flow and protects against ischemia/reperfusion injury (107,110–114). NO also scavenges superoxide to form peroxynitrite (112). The resulting decrease of superoxide may be a beneficial effect; however, peroxynitrite is itself a toxic radical. In general, NO production protects against ischemia/reperfusion injury.

HEPATIC NEUTROPHIL INFILTRATION AFTER ISCHEMIC/REPERFUSION INJURY

Chemoattractant molecules produced in large part by Kupffer cells cause hepatic neutrophil infiltration, which mediates the late phase of hepatic ischemia/reperfusion injury. Infiltrating neutrophils perpetuate and amplify injury by releasing many of the same mediators (e.g., reactive oxygen species, cytokines) as Kupffer cells, but often in much larger quantities. Antineutrophil antibodies decrease hepatocellular necrosis 24 hours after hepatic ischemia/reperfusion from 80% to 28% (115).

Neutrophils marginate to the sinusoidal wall and then migrate across the endothelium into the space of Disse. Margination first involves binding of neutrophils to selectin molecules on sinusoidal endothelial cells (116). If endothelial cell damage is severe, transendothelial neutrophil migration occurs easily. Otherwise the interaction of intercellular adhesion molecules (ICAMs) such as ICAM-1 with integrins such as CD11b/CD18 (also called MAC-1) on neutrophils occurs to enhance both adherence and migration through the endothelial lining (see Chapters 32 and 33 and website chapters W-27 and W-28). TNF-α, IL-1, and interferon-γ stimulate ICAM-1 expression on hepatocytes and endothelial cells, and anti–ICAM-1 antibodies decrease leukocyte adherence and diminish late hepatic ischemia/reperfusion injury even when added 8 hours after reperfusion (117,118). After warm ischemia/reperfusion, neutrophil CD11b/CD18 expression is upregulated, and antibodies against CD11b attenuate hepatic neutrophil infiltration, superoxide formation, and hepatocellular injury after ischemia/reperfusion (89,119). When neutrophils infiltrate the liver, they worsen hepatic hypoperfusion, exacerbate the effects of ET-1, and may release proteases (cathepsin G, granulocytes elastase) that are toxic to hepatocytes (120,121). Infiltrating neutrophils also produce toxic reactive oxygen species, including superoxide and hydroxyl radicals.

T lymphocytes also play a role in the late phase of hepatic ischemia/reperfusion injury. T-lymphocyte–deficient and $CD4^+$-depleted mice but not $CD8^+$-depleted mice have decreased neutrophil infiltration, liver enzyme release, and hepatocellular necrosis late after hepatic ischemia/reperfusion. $CD4^+$ cells (T-helper cells) infiltrate the liver within an hour after reperfusion and likely provide another signal for neutrophil infiltration (122).

EARLY, INTERMEDIATE, AND LATE PHASES OF REPERFUSION INJURY

Different mechanisms mediate the early, intermediate, and late phases of hepatic ischemia reperfusion injury (Table 18.2). The early phase involves pH-dependent mitochondrial dysfunction and leads to hepatocellular necrosis within minutes of reperfusion and perhaps apoptosis after longer times. Kupffer cell production of free radicals, cytokines, and chemokines principally mediate the intermediate phases of reperfusion injury for up to 6 hours after restoration of blood flow. The late, neutrophil-mediated phase of hepatic ischemia/reperfusion injury involves margination and transendothelial migration of neutrophils in response to Kupffer cell–generated chemoattractants and release of toxic mediators, such as reactive oxygen species and proteases. These three phases of reperfusion injury take on differing importance depending on the length of

TABLE 18.2. PHASES OF HEPATIC ISCHEMIA/REPERFUSION INJURY

Ischemia/anoxia
Necrotic cell death beginning after 30 min anoxia/ischemia at pH 7.4
ATP depletion–dependent
Protection by glycolysis, glycine, and pH <7
ROS at anoxic/normoxic border
Phospholipase and protease activation
Reperfusion injury
Early phase
Necrotic cell death within 1 h of reperfusion
Large component of pH-dependent (pH >7), ROS-independent cell killing
Mitochondrial permeability transition
Kupffer cell activation and ROS formation
Microcirculatory disturbances
Intermediate phase
Kupffer cell–mediated necrosis and apoptosis after 6 or more h
Kupffer cell release of ROS, cytokines, and chemokines
TNF-α–mediated lung injury
Kupffer cell–initiated neutrophil margination
T-helper cell infiltration
Microcirculatory disturbances
Late phase
Neutrophil-mediated necrosis and apoptosis after up to 24 h
Neutrophil transendothelial migration, activation, and release of ROS, cathepsin, and elastase
Microcirculatory disturbances

ROS, reactive oxygen species; TNF, tumor necrosis factor.

ischemia. After prolonged severe ischemia, the immediate phase predominates. After less severe ischemia, the intermediate and late phases gain more relative importance.

Ischemia/reperfusion injury of the liver can lead to multisystem organ failure. Cytokines and chemokines released by activated Kupffer cells likely promote the pulmonary edema and interstitial infiltration of leukocytes observed after hepatic ischemia/reperfusion. Mononuclear cells in the marginal zones of the spleen also increase, and splenectomy before ischemia/reperfusion decreases neutrophil infiltration into the liver and hepatocellular injury (123). Thus, a systemic inflammatory response involving the spleen also appears to promote liver injury.

ISCHEMIC PRECONDITIONING

Brief periods of myocardial ischemia followed by reperfusion render both human and animal hearts resistant to subsequent prolonged ischemia (124). Such ischemic preconditioning decreases infarct size after subsequent long ischemia and reperfusion. Ischemic preconditioning also protects a variety of other organs, including the liver (125,126). Ischemic preconditioning of the liver decreases hepatocellular enzyme release and mortality after warm ischemia and reperfusion, an effect mediated in part by increased NO formation and heat shock protein synthesis (127,128).

During ischemia, ATP quickly degrades to adenosine. This release is another important mediator of ischemic preconditioning, and in the liver adenosine suppresses TNF-α release from Kupffer cells (129). Adenosine has three types of cellular receptors that differ in their biochemical and pharmacologic responses to adenosine agonists and antagonists (130). Activation of adenosine A_1 and A_3 receptors stimulates inhibitory G proteins that block adenylyl cyclase and decrease 3′,5′-cyclic adenosine monophosphate (cAMP). By contrast, adenosine A_2 receptor activation stimulates adenylyl cyclase and increases cAMP. In the heart, the adenosine A_1 receptor pathway mediates ischemic preconditioning of myocardium, whereas adenosine A_2 receptors mediate ischemic preconditioning of coronary endothelial cells (131,132). In the liver, adenosine A_2 receptors mediate ischemic preconditioning of hepatocytes against warm ischemia/reperfusion by stimulating NO synthesis (133). In the heart, a potential downstream target of adenosine receptor activation is the mitochondrial K_{ATP} channel, and K_{ATP} channel openers such as cromakalin confer protection against ischemic injury (134). The effect of K_{ATP} blockers and openers on hepatic ischemia/reperfusion injury has not yet been studied.

LIVER PRESERVATION FOR TRANSPLANTATION SURGERY

Liver transplantation is the only therapy for children and adults with end-stage liver disease that provides long-term survival and resumption of a normal life style. Using University of Wisconsin (UW) cold storage solution (Table 18.3), donor livers from brain-dead heart-beating cadaver donors can be preserved successfully by simple cold ischemia for up to 24 hours (135,136). Longer preservation times are limited by a reperfusion injury that occurs predominantly to sinusoidal endothelial and Kupffer cells (Fig. 18.9). A consequence of inadequate preservation is primary graft nonfunction, which still occurs in 5% to 10% of patients (137,138). Metabolic failure of the newly implanted graft, rapidly rising serum transaminases, lack of bile formation, and severe coagulopathy characterize primary nonfunction. Primary nonfunction progresses quickly to hepatic encephalopathy, acute renal failure, disseminated intravascular coagulation, and death unless retransplantation is performed within 3 to 4 days. Since donor livers are in very short supply, every retransplantation due to primary graft nonfunction means another patient on the waiting list dies. For this reason, the continuing need exists to develop new approaches to prevent graft failure from preservation injury and to expand the donor pool to include marginal donor livers, such as fatty livers and livers from non–heart-beating cadavers, which are now considered too risky to use.

Hepatic injury also occurs to grafts that survive and eventually perform well. Serum transaminases typically rise sharply 1 to 3 days postoperatively, which is sometimes followed by a syndrome of primary graft dysfunction or initial poor function characterized by functional cholestasis with sustained high bilirubin, diminished bile flow, and centrilobular hepatocellular ballooning and feathery degeneration (139). Moderate to severe primary graft dysfunction occurs in up to 30% of liver transplant recipients and is associated with two- to fourfold greater overall graft loss, longer intensive care unit and hospital stays, and increased overall mortality (137–140). Nonanastomotic biliary strictures can also develop in transplant patients beginning after

TABLE 18.3. UNIVERSITY OF WISCONSIN (UW) COLD PRESERVATION SOLUTION

Ingredient	Concentration
K-lactobionate	100 mM
Na KH_2PO_4	25 mM
Adenosine	5 mM
$MgSO_4$	5 mM
Glutathione	3 mM
Raffinose	30 mM
Allopurinol	1 mM
Hydroxyethyl starch	50 g/L
Dexamethasone	8 mg/L
Insulin	100 U/L
Bactrim	0.5 ml/L
Total Na^+	30 mM
Total K^+	120 mM
pH	7.4
Osmolarity	310–330 mOsm

several weeks (141). The incidence of strictures rises with more prolonged storage times, suggesting a role of preservation injury in their etiology. The clinical incidence of primary graft nonfunction and dysfunction and of ischemic biliary strictures is dependent on time of storage. For example, primary nonfunction and early retransplantation occur nearly four times more often after 20 hours or more of storage than after less than 10 hours. Fatty livers from alcoholic and obese donors tend to do poorly after transplantation (142). In the absence of steatosis, however, metabolic tests and histology of biopsies taken just prior to reperfusion do not discriminate viable grafts from nonviable grafts. Such tests are unable to predict graft performance because the critical injury leading to liver graft failure is a reperfusion injury after storage (143).

ENDOTHELIAL CELL DAMAGE FROM STORAGE/REPERFUSION INJURY

Endothelial cells line the hepatic sinusoids to form a fenestrated sieve plate separating the vascular space from the subsinusoidal surface of the hepatocytes (see Chapters 30 and 31 and website chapters W-26 and W-32). During cold ischemic storage, endothelial cells round up and retract their extended sheet-like cytoplasm (144,145). After shorter periods of storage, these changes are reversible, and the rounded endothelial cells spread out after warm reperfusion. However, after times of cold storage that result in graft failure after transplantation, warm reperfusion leads to the destruction of the sinusoidal lining (Figs. 18.9 and 18.10) (reviewed in ref. 8). Reperfusion initiates a sequence of nuclear membrane vacuolization, mitochondrial swelling and lysis, ball-like rounding, plasma membrane breaks, cytoplasmic rarefaction, and nuclear condensation. Shredded fragments are all that remain of the sieve plates, and virtually all endothelial cell nuclei label with trypan blue, an indicator of loss of cell viability (Fig. 18.10). Release of the BB isozyme of creatine kinase, an enzyme localized to hepatic nonparenchymal cells, also indicates loss of endothelial cell viability. Similar changes to endothelial cells occur in human livers after storage and reperfusion. Kupffer, stellate (fat-storing or Ito), and bile duct epithelial cells, however, retain viability after cold storage and reperfusion.

Because hepatic sinusoidal endothelial cells are responsible for the clearance of hyaluronic acid from the plasma, levels of serum hyaluronic acid and hepatic clearance of hyaluronic acid provide useful indices of sinusoidal endothelial cell function and viability *in vivo* (146). After cold storage and transplantation of rat livers, decreases of hyaluronic acid clearance and increases of serum hyaluronic acid parallel loss of endothelial cell viability (147,148). Hyaluronic acid content in the initial effluent after reperfusion of stored human livers and measurement of serum hyaluronic acid and hepatic hyaluronic acid clearance are early predictors of liver graft function in clinical transplantation (148,149).

Liver preservation in UW cold storage solution decreases lethal reperfusion injury to endothelial cells after longer periods of cold storage compared to other preservation solutions in current or previous use, including Euro-Collins solution, HTK (*h*istidine–*t*yrptophane–α-*k*etoglutarate solution), and Celsior solution (150,151). The improvement of endothelial cell viability after reperfusion parallels the improvement of graft survival after transplantation. Although hepatic damage after warm anoxic injury is much

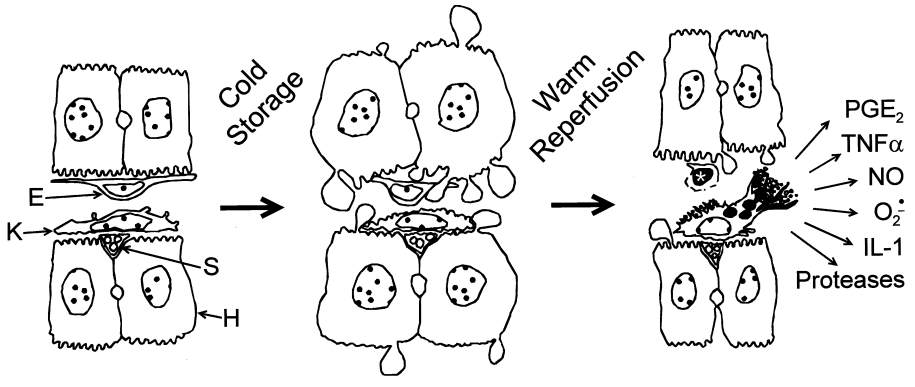


FIGURE 18.9. Reperfusion injury to liver after cold ischemic preservation. During cold ischemic storage, hepatocytes (*H*) swell and form cell surface blebs. Moderate rounding of sinusoidal endothelial cells (*E*) and Kupffer cells (*K*) also occurs. Stellate cells (*S*) change little in structure. After warm reperfusion, endothelial cells lose viability, and their nuclei stain with supravital dyes like trypan blue (*asterisk*). Also after reperfusion, Kupffer cells swell, ruffle, and degranulate. These activated Kupffer cells release inflammatory mediators, including prostaglandin E_2 (PGE_2), TNF-α, nitric oxide (*NO•*), superoxide radical ($O_2^{\bullet-}$), interleukin-1 (*IL-1*), and proteases. Hepatocytes recover after reperfusion by resorbing blebs and recovering volume regulation. Stellate cells remain relatively undisturbed. (Adapted from Lemasters JJ, Thurman RG. Reperfusion injury after liver preservation for transplantation. *Annu Rev Pharmacol Toxicol* 1997;37:327–338.)

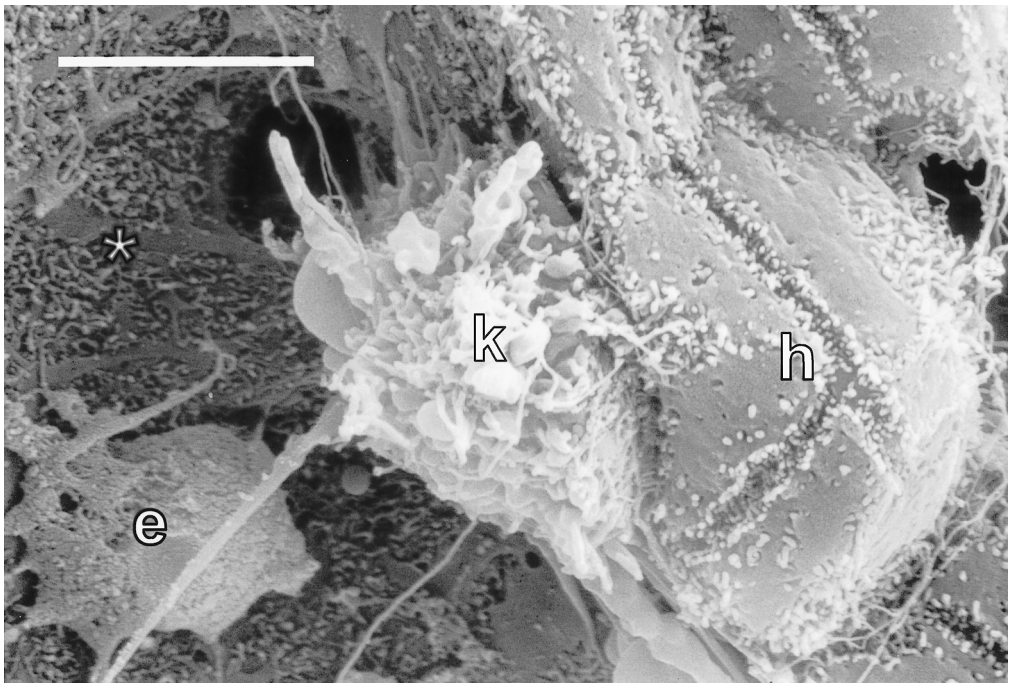


FIGURE 18.10. Scanning electron micrograph of Kupffer cell activation and endothelial cell killing after cold storage and warm reperfusion. A Kupffer cell (*k*) shows swelling, rounding, and ruffling after 24 hours cold storage of a rat liver in Euro-Collins solution and 15 minutes of warm reperfusion. The endothelial lining of the sinusoid is denuded (*asterisk*), and only a remnant of an endothelial cell (*e*) remains. Hepatocytes (*h*) show normal features. *Bar* is 5 μm. (Adapted from Caldwell-Kenkel JC, Currin RT, Tanaka Y, et al. Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. *Hepatology* 1991;13:83–95.)

decreased in livers from fed rats compared to fasted ones because of glycogen accumulation in hepatocytes (24,57), storage/reperfusion injury to endothelial cells is the same in fed and fasted rats (144). However, glycogen superloading of livers appears to decrease endothelial cell injury (152). Protection may be mediated by glucose release from glycogen-loaded hepatocytes, since glucose protects endothelial cells against hypoxic killing (15).

KUPFFER CELL ACTIVATION AFTER COLD STORAGE AND REPERFUSION

During cold ischemic liver storage, relatively minor structural changes occur to Kupffer cells, notably some rounding and blunted ruffling (145). In contrast to these minor changes, warm reperfusion after storage initiates a rapid and marked activation of Kupffer cells (Figs. 18.9 and 18.10) (144,145). Structural changes indicative of activation include vacuolization, cell surface ruffling, formation of wormlike densities, and degranulation within minutes after reperfusion. Cold storage and reperfusion also activate a variety of functional activities in Kupffer cells, including particle phagocytosis, superoxide formation, and hydrolytic enzyme release (145,153,154).

Kupffer cell activation and endothelial cell killing occur in parallel after reperfusion, and both are diminished by storage in UW solution (144,145). These perturbations to nonparenchymal cells are also documented *in vivo* in rat liver grafts that are failing from storage/reperfusion injury (155,156), as well as in human clinical specimens (157,158). Kupffer cells activated by storage/reperfusion injury release TNF-α after liver transplantation, which promotes edema and leukocyte infiltration in the lung (159–162).

REVERSIBLE HEPATOCELLULAR CHANGES DURING LIVER PRESERVATION

During cold ischemic storage, hepatocytes swell and form surface blebs much like those formed during warm hypoxia/ischemia (144,145,163). After longer periods of storage, such blebs literally fill the sinusoids. Hepatocellular swelling and blebbing are less severe after shorter periods of storage, and both occur more slowly during storage in UW solution. Even macroscopically, swelling is much less marked in livers stored in UW solution compared to Euro-Collins solution. Reperfusion after cold storage reverses hepatocellular bleb formation and swelling, and little hepatocellular

death and lactate dehydrogenase (LDH) release occur, even after 96 hours of cold storage in Euro-Collins solution, long past the point when liver grafts fail from storage/reperfusion injury (164). Similarly, hepatocellular oxygen consumption and carbohydrate metabolism remain within normal limits (144,164,165). Thus, damage to hepatocytes seems not to underlie liver storage/reperfusion injury.

MICROCIRCULATORY DISTURBANCES AND FREE RADICAL GENERATION AFTER REPERFUSION OF STORED LIVERS

After cold ischemic liver storage, reperfusion with blood leads to microcirculatory disturbances characterized by leukocyte margination, platelet adhesion, fibrin deposition, inflammation, and hemostasis that increases with increasing time of storage (156,166–168). In normal untransplanted livers, leukocyte movement in hepatic sinusoids is rapid and continuous with almost no margination (156,169). In liver grafts failing storage/reperfusion injury, leukocyte velocity decreases substantially, and leukocyte margination increases from virtually 0 to 40% of cells. Kupffer cell phagocytosis is also enhanced. Subsequently, hepatocytes lose viability beginning about 4 hours after transplantation. Even in liver grafts that survive, microcirculatory disturbances occur after reperfusion that are associated with foci of hepatocellular necrosis 24 hours later. Consistent with these experimental findings, platelet trapping in human liver grafts predicts poorer outcomes in clinical liver transplantation (158,170).

Activated Kupffer cells release a variety of inflammatory mediators directly into the blood, including superoxide radicals, NO, proteases, eicosinoids, TNF-α, and other cytokines. These mediators intensify the inflammatory responses and microcirculatory disturbances already created by damage to the sinusoidal endothelium. Spin trapping techniques and nitroblue tetrazolium cytochemistry document free radical formation by Kupffer cells after storage and reperfusion (154,171). Free radicals help stimulate neutrophil margination after reperfusion, since superoxide dismutase decreases neutrophil infiltration into reperfused liver after both warm and cold ischemia (172,173). The systemic release of inflammatory mediators also promotes the adult respiratory distress syndrome and multiple organ failure associated with liver failure (174). Treatments that activate Kupffer cells, such as donor treatment with lipopolysaccharide or physical manipulation of the explanted liver, decrease graft survival dramatically (175–177). Conversely, treatments that suppress Kupffer cell activity, including L-type voltage-sensitive calcium channel blockers, pentoxifylline, adenosine, and prostaglandin E_1, improve graft survival (178–180). Adenosine and prostaglandin E_1 act by cAMP-dependent receptor mechanisms (129), whereas pentoxifylline is a phosphodiesterase inhibitor that blocks cAMP hydrolysis.

Kupffer cell degranulation after cold storage and reperfusion leads to the release of hydrolytic enzymes, including proteases, and the activity of the calcium-dependent protease calpain increases in liver tissue after cold storage and warm reperfusion (181). Free amino acids in the initial reperfusion effluents of stored livers also increase with increasing times of storage (182). Protease inhibition, however, fails to improve long-term graft survival, although some temporary benefit may result (153,183). Thus, protease activation alone does not explain graft failure after storage/reperfusion injury. Rather, a combination of mediators released from Kupffer cells likely promotes graft failure, including reactive oxygen species, proinflammatory cytokines, hydrolytic enzymes such as proteases, and possibly other toxic mediators.

Kupffer cell activation does not cause endothelial cell killing after storage/reperfusion, because Kupffer cell inactivation by pretreatment with $GdCl_3$ fails to decrease endothelial cell killing (184). Similarly, antioxidants, allopurinol, desferal, superoxide dismutase, and catalase, and washout with anoxic buffer have no benefit in preserving endothelial cell viability (154). Thus, oxygen-independent mechanisms mediate lethal storage/reperfusion injury to sinusoidal endothelial cells. Nonetheless, subsequent oxygen-dependent events following endothelial injury involving Kupffer cells contribute to graft failure from storage/reperfusion injury. One consequence is neutrophil infiltration and subsequent neutrophil-mediated hepatic injury by the same mechanisms that occur after warm ischemia/reperfusion injury.

MITOCHONDRIAL CHANGES AND APOPTOSIS FROM STORAGE/REPERFUSION INJURY

Lethal storage/reperfusion injury to sinusoidal endothelial cells is highly pH-dependent, and reperfusion of stored livers with acidotic buffer (pH 6.5 to 6.8) greatly decreases endothelial cell killing (154). The mitochondrial permeability transition may contribute to this pH-dependent injury, since cyclosporin A decreases sinusoidal endothelial cell killing after warm anoxia/reoxygenation (185). Cyclosporin A also decreases reperfusion injury to livers after cold ischemia (186). pH-dependent hypoxic killing of sinusoidal endothelial cells is also associated with mitochondrial depolarization (15). However, cyclosporin A alone does not always prevent the endothelial cell killing associated with warm hypoxia and cold ischemia/reperfusion. Similarly, in isolated mitochondria and hepatocytes, cyclosporin A alone may not be sufficient to prevent the mitochondrial permeability transition after prolonged exposure to strong permeability transition inducers.

Apoptosis to both parenchymal and nonparenchymal cells is also a feature of storage/reperfusion injury and

increases with increasing times of cold ischemic storage (187,188). Such apoptosis peaks about 12 hours after transplantation in nonparenchymal cells and after 48 hours for parenchymal cells. A tenfold increase of apoptosis can occur in the absence of primary graft failure and may underlie delayed oxygen-dependent parenchymal cell killing in surviving liver grafts. Activation of caspase 3 and other proteases accompanies apoptosis after storage/reperfusion injury, and the caspase inhibitor IDN-1965 decreases apoptosis by nearly two-thirds (189,190). However, IDN-1965 and other protease inhibitors do not improve long-term graft survival (153,190). Thus, apoptosis may be a consequence rather than a cause of storage/reperfusion injury.

RINSE STRATEGIES TO DECREASE STORAGE/REPERFUSION INJURY

If reperfusion injury is a major contributor to liver graft failure, then modification of the conditions of reperfusion might decrease this injury and improve graft performance and viability. To this end, a new solution was devised at the University of North Carolina to counter potential mechanisms contributing to reperfusion injury (Table 18.4) (191). This solution, named Carolina rinse solution, contains electrolytes similar to Ringer's solution, antioxidants (glutathione, desferal, allopurinol), substrates to regenerate ATP (fructose, glucose, insulin, and adenosine), a calcium channel blocker (nicardipine), colloid osmotic support against interstitial edema (hydroxyethyl starch), a cytoprotective amino acid (glycine), and mildly acidic pH (MOPS buffer, pH 6.5). Carolina rinse solution blocks both reperfusion-induced endothelial cell killing after liver storage and the activation of Kupffer cells (192). Carolina rinse solution also improves early bile flow and microvascular perfusion and decreases leukocyte infiltration (192,193). Most significantly, Carolina rinse solution substantially decreases liver graft failure from storage/reperfusion injury when it is flushed into liver explants at the end of storage (194,195). Controversy has existed over whether the injury causing liver graft failure after prolonged cold ischemia in stored livers is a reperfusion injury, or whether irreversible damage occurs during storage prior to reperfusion. The efficacy of Carolina rinse solution used at the end of storage is perhaps the strongest evidence that the injury causing graft failure is a reperfusion-induced event. Two small randomized prospective clinical trials confirm the efficacy of Carolina rinse solution to decrease graft injury after liver transplantation (196,197). However, these pilot studies await confirmation in a large multicenter trial.

TABLE 18.4. CAROLINA RINSE SOLUTION

Ingredient	Concentration
NaCl	115 mM
KCl	5 mM
$CaCl_2$	1.3 mM
KH_2PO_4	1 mM
$MgSO_4$	1.2 mM
Hydroxyethyl starch	50 g/L
Allopurinol	1 mM
Desferrioxamine	1 mM
Glutathione	3 mM
Fructose	10 mM
Glucose	10 mM
Glycine[a]	5 mM
Adenosine	200 μM
Nicardipine	2 μM
Insulin	100 U/L
3-[*N*-morpholino]propanesulfonic acid (MOPS)	20 mM
Total Na^+	115 mM
Total K^+	6 mM
Total Cl^-	122
pH	6.5
Osmolarity	290–305 mOsm

[a]Early versions of Carolina rinse solution did not contain glycine.

Several of the individual components of Carolina rinse solution have been shown to contribute to its efficacy. Adenosine at an optimal concentration of 100 to 200 μM is needed for maximal efficacy, and graft survival decreases when adenosine concentration is either increased or decreased (192,194,195). Antioxidants (glutathione, allopurinol, desferal) and acidic pH (pH 6.5) also are required for efficacy (162). Lastly, the addition of glycine to the other components also improves graft survival after rat liver transplantation (198). The remaining components of Carolina rinse solution, although not specifically demonstrated to contribute to better graft survival, act to counter specific mechanisms contributing to storage/reperfusion injury. The calcium channel blocker (nicardipine) and adenosine suppress lipopolysaccharide-stimulated TNF-α release by cultured Kupffer cells (129,179), and suppression of Kupffer cell activation is one of the likely mechanisms by which Carolina rinse solution improves graft survival after storage/reperfusion injury. Adenine and ribose, the metabolic precursors of adenosine, cannot replace adenosine in Carolina rinse solution (194), supporting the conclusion that adenosine acts via a receptor, possibly adenosine A_2 receptors on both Kupffer cells and endothelial cells. Adenosine also has vasodilatory effects that improve the hepatic microcirculation after reperfusion. As described above, fructose and glucose are substrates for glycolytic ATP formation that protect hepatocytes and sinusoidal endothelial cells, respectively, against hypoxic cell killing.

The efficacy of Carolina rinse solution is also increased when it is used at a warm (30° to 37°C) rather than a cold (1° to 4°C) temperature (195). One benefit of warm temperature is improved microcirculation and decreased vascular resistance to permit more rapid and homogenous reperfusion (159,199). In hepatocytes treated with cyanide to mimic anoxia-induced ATP depletion during organ storage,

the rank order of loss of cell viability in various solutions at 0° to 1°C is Ringer's solution > Carolina rinse solution > UW solution (200). However, as temperature increases above 12°C, the rank order of cell killing in different solutions changes to UW solution > Ringer's solution >> Carolina rinse solution. These findings illustrate how UW solution is optimized to be cytoprotective at low temperatures, whereas Carolina rinse solution is optimally effective at warm physiologic temperatures.

ISCHEMIC PRECONDITIONING OF LIVERS PRIOR TO STORAGE

As after warm ischemia, ischemic preconditioning decreases graft injury and improves graft survival after cold ischemic storage of livers. Specifically, ischemic preconditioning prior to storage decreases endothelial cell killing, Kupffer cell activation, and liver graft failure after prolonged cold preservation (201–203). Adenosine mediates this cytoprotection by a cAMP-coupled adenosine A_2 receptor mechanism (184). Similarly, prostaglandin E_2 stimulates Kupffer cells and sinusoidal endothelial cells by cAMP-linked prostaglandin receptors, and donor pretreatment with prostaglandin analogues such as dimethylprostaglandin E_2 decreases endothelial cell killing after cold storage/reperfusion and improves graft success after transplantation (204). These findings in experimental animals hold promise that pretreatment of human donors prior to organ harvest might substantially improve graft function and viability after clinical transplantation.

CONCLUSION

Ischemia/reperfusion involves several different hepatic cell types and involves a variety of mechanisms. During prolonged warm anoxia and ischemia, hepatocellular killing occurs by pH-dependent but oxygen-independent mechanisms. After reperfusion, early hepatocellular killing is also highly pH-dependent and involves a specific injury to mitochondria called the mitochondrial permeability transition. Ischemia/reperfusion also causes Kupffer cell activation, which leads to formation of reactive oxygen species, release of cytokines and chemoattractants, and the recruitment of neutrophils, events that promote liver injury up to 24 hours after reperfusion. Cold ischemia/reperfusion associated with liver preservation for transplantation surgery also involves activation of Kupffer cells, but pH-dependent endothelial cell killing follows reperfusion instead of hepatocyte killing. Studies of hepatic ischemia/reperfusion injury show that multiple mechanisms contribute to hepatic damage. Only by combating these multiple mechanisms of injury can effective strategies be developed to prevent reperfusion injury after warm ischemia and cold ischemic storage.

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