Role of Apoptosis in Hypoxic/Ischemic Damage in the Kidney

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Cell death by hypoxia/ischemia may occur by apoptosis as well as necrosis in experimental models of renal injury both in vivo and in vitro. Necrosis can occur during hypoxia/ischemia as a result of widespread cellular degradation, and during reoxygenation/reperfusion as a consequence of the development of the mitochondrial permeability transition pore (PTP). In vitro models of hypoxia/reoxygenation suggest that apoptotic cell death may occur during reoxygenation as a consequence of mitochondrial release of cytochrome *c* (Cyt *c*) during hypoxia. In hypoxic renal cells, Bax and Bak, 2 pro-apoptotic proteins of the Bcl-2 family, collaborate to permeabilize the mitochondrial outer membrane to intermembrane proteins such as Cyt *c*, although Bax, per se, appears to play the dominant role. Cyt *c* then acts to trigger the downstream apoptotic cascade. Caspase inhibitors suppress these downstream events, but not Cyt *c* release. However, the anti-apoptotic Bcl-2 prevents mitochondrial permeabilization and maintains viability. Inflammation is known to play a major role in exacerbating parenchymal damage during reperfusion. Recent studies suggest that the apoptosis-related mechanisms contribute to the inflammatory process. By inhibiting tubular cell apoptosis, by suppressing an apoptotic chain reaction in accumulating inflammatory cells, and by inhibiting caspase-1 processing in injured tissue, caspase inhibitors may reduce inflammation, and thereby reduce the cascading parenchymal injury that is associated with inflammation.

ISCHEMIA CAUSED BY arterial occlusion, shock, or transplantation often leads to kidney failure as a result of increased cell death in the affected kidneys. Ischemic kidneys suffer profound losses of adenosine triphosphate (ATP) owing to reduced availability of oxygen and nutrients causing inhibition of both oxidative phosphorylation as well as anaerobic glycolysis. For this reason, ischemia per se causes more cell damage than hypoxia, which still permits anaerobic glycolysis. Some cells die during ischemia itself, mostly by the necrotic form of cell death in which early membrane damage leads to activation of multiple degradative systems in an uncontrolled fashion. Necrosis is observed predominantly in proximal tubules of the kidney, which use mitochondrial respiration as the sole source for their energy requirements.[1] Therefore, these segments of nephron are more prone to ischemic insults than other regions of nephron during the initial stages of ischemic insults. Of much clinical and scientific interest is the additional loss of kidney parenchymal cells that occurs during reperfusion after ischemia. Latent, but potentially lethal, ischemic damage may cause cells in different regions of kidney to undergo reperfusion injury when reoxygenated by blood reflow. Because availability of oxygen and nutrients during reperfusion allows ischemic cells to restore ATP pools, additional cell loss during reperfusion could be apoptotic because this form of cell death requires energy.[2] In addition, cell death also can be caused secondarily by inflammation around dead tissue. In fact, a deleterious role of the inflammatory response was shown in ischemia/reperfusion-induced kidney damage.[3] The inflammatory response mainly was owing to enhanced local expression of adhesion molecules and pro-inflammatory mediators that activated the neutrophils and the complement system.[4]

Reperfusion injury also has been attributed to the toxic effects of free radicals and other reactive metabolites generated by the direct reduction of oxygen by electrons diverted from respiratory pathways. Although reperfusion does lead to overproduction of free radicals, there is little evidence that radicals produced by the parenchymal cells are associated with death mechanisms. After an initial increase, cellular concentrations of radicals are buffered sharply down by endogenous antioxidants. On the other hand, invading inflammatory cells do add an element of oxidant damage to reperfusion injury. In one series of studies, defects of mitochondrial electron conduction and oxidative phosphorylation that persist during reoxygenation were proposed to be the triggering mechanisms that operate within parenchymal cells and cause reperfusion injury.[5-7] In these studies, respiratory

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defects were shown at the level of complex I of the electron transport chain.[5] On the other hand, loss of cytochrome *c* (Cyt *c*) caused by permeabilization of the outer membranes may be another reason for respiratory dysfunction in postischemic mitochondria. In support, electron transport was restored by the addition of Cyt *c* to mitochondria isolated from ischemic hearts.[8] Such studies have been open to questions whether the leaks of Cyt *c* had indeed occurred in vivo or had been induced artifactually by trauma associated with the isolation procedure. Investigation of death pathways operative during disease in vivo is important, but technically difficult owing to variables such as heterogeneity of cell types in organs, differences between organ systems, and exacerbation of tissue damage caused by inflammation. In view of these considerations, studies using purified cells in culture will offer advantages toward arriving at a better understanding of hypoxic/ischemic injury at the molecular level.

APOPTOSIS VERSUS NECROSIS

Apoptosis and necrosis originally were described to be 2 distinct forms of cell death, which can be distinguished clearly.[9] Necrosis may occur in response to diverse noxious stimuli including toxins, physical stimuli, or ischemia, as a result of severe breakdown in cellular homeostasis. Swelling of cells and disruption of membranes are prominent features of necrosis, and the nuclear chromatin undergoes lysis (karyolysis), not condensation, in this mode of cell death. In necrotic cells, cellular organelles characteristically are swollen. In contrast, apoptotic cells are shrunken and develop protrusions containing dense cytoplasm. Membrane integrity is not lost until late, after cell death. Nuclear chromatin undergoes striking condensation and fragmentation. The cytoplasm becomes divided to form apoptotic bodies containing organelles and/or nuclear debris. Because large groups of cells are involved and cellular contents are lost early into the extracellular space, necrotic tissues evoke vigorous inflammatory responses. In contrast, membrane damage occurs late during apoptosis, and neighboring cells or phagocytes engulf dead cells leading to little or no inflammation. Although physiologic deletion of cells by apoptosis occurs during development, homeostatic regulation of cell populations and aging, pathologic cellular demise imposed by toxins, physical agents, or ischemia also may induce typical apoptotic morphology. Unlike apoptosis, necrosis does not require energy for its occurrence; and stimuli that induce apoptosis in the presence of energy lead to necrosis in the absence of energy owing to a severe decline in cell ATP levels.[2,10]

However, these 2 types of cellular demise can occur simultaneously in tissues or cultured cells exposed to ischemia/reperfusion or hypoxia/reoxygenation, respectively.[11-15] Moreover, the intensity and duration of insults may decide the cell death outcome.[16,17] Thus, in certain cases, triggering events can be common for both types of cell death. Probably, for an organized execution of apoptosis, a downstream controller may be required. For any reason, if the apoptotic program is aborted before this control point, and the initiating stimulus is severe, cell death still may result by necrosis.[18] This view is supported by the finding that although caspase inhibition was sufficient to prevent proteolysis and DNA fragmentation during apoptosis initiated by mitochondrial permeabilization, it did not prevent ultimate cell death by necrosis.[19] The inability of caspase inhibitors to confer long-term viability under these circumstances could be related to functional injury of mitochondria.[10,19,20] Apoptotic processes were shown to involve at least 2 different mechanisms initiated by distinct caspases. In apoptosis mediated by death receptors, ligated receptors recruit and proteolytically activate the initiator caspase-8,[21] which in turn triggers effector caspases. Because the proximate trigger involves the initiator caspase, caspase inhibitors abort the entire death cascade and provide complete protection. However, other apoptotic stimuli lead to the activation of caspase-9 and require a proximate trigger that involves increased mitochondrial permeability and release of Cyt *c*.[22,23] In this case, in contrast to receptor-mediated apoptosis, caspase inhibitors cannot prevent irreparable mitochondrial damage, thus assuring cell death that might be expressed as necrosis.[10,19,24,25]

IN VITRO MODEL FOR ISCHEMIA/REPERFUSION INJURY

To understand the molecular basis of reperfusion/reoxygenation injury, in vitro models of cultured rat kidney proximal tubule cells were developed.[10,26] The results from these studies showed that reoxygenation of hypoxic cultured proximal tubule cells by re-incubation in growth medium

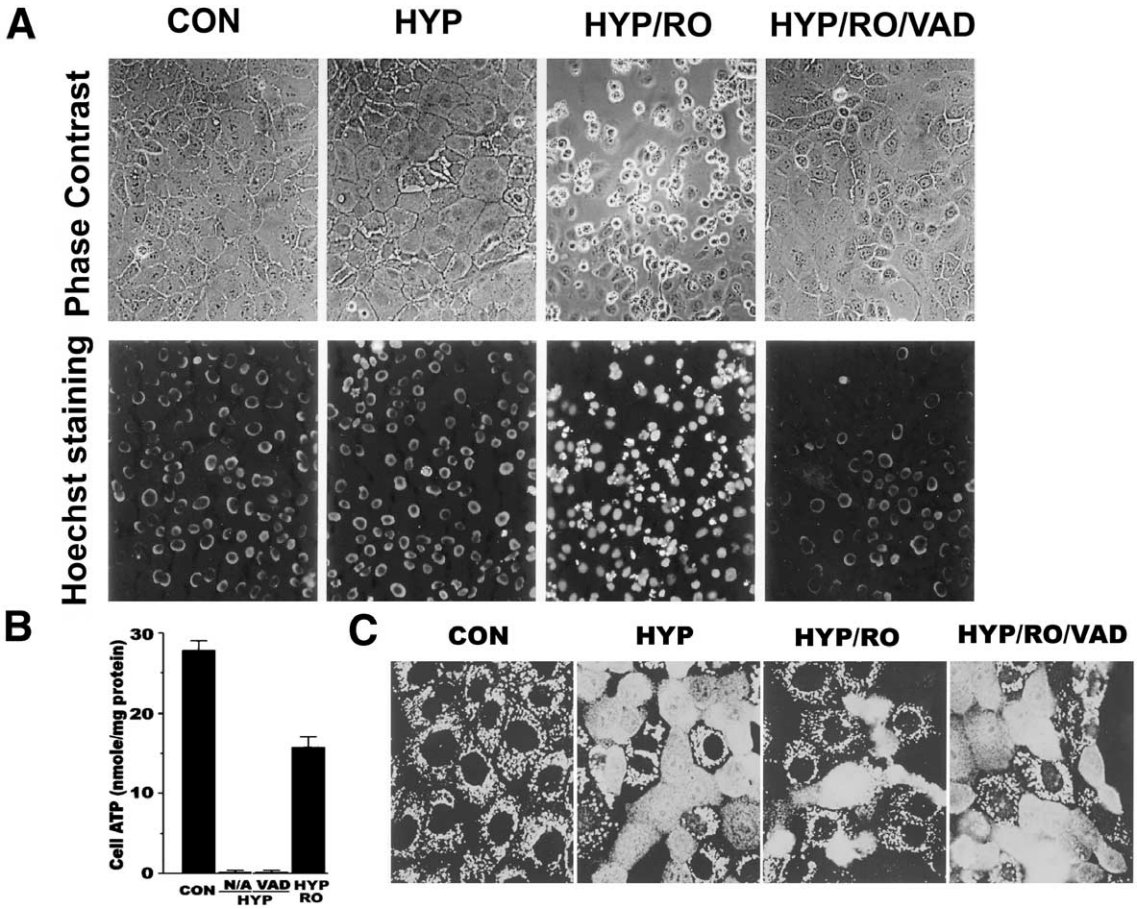

Fig 1. Effect of hypoxia and reoxygenation on rat kidney proximal tubule cells in vitro. Cells were incubated in glucose and serum-free medium for 5 hours in an anaerobic chamber (95% N_2/5% CO_2) for hypoxia and replaced with complete growth medium with glucose and serum and incubated under normoxic conditions for reoxygenation. (A) Morphologic features of control (CON), hypoxic (HYP), and reoxygenated (HYP/RO) proximal tubule cells stained with Hoechst 33258 and were observed by phase contrast microscopy or fluorescence microscopy. Reoxygenated cells (HYP/RO) but not hypoxic cells (HYP) show morphologic features characteristic of apoptosis. A broad-spectrum inhibitor of caspase enzymes, z-VAD, inhibited reoxygenation injury (HYP/RO/VAD). (B) Cellular ATP levels measured in control (CON), hypoxic (N/A), and reoxygenated cells (HYP/RO) show that the ATP depletion that occurs during hypoxia is not ameliorated by z-VAD (VAD). During reoxygenation (HYP/RO), ATP levels are increased owing to glycolysis. (C) Immunofluorescence analysis shows that Cyt *c* is released during hypoxia. Cells that leaked Cyt *c* undergo apoptosis during reoxygenation. Inclusion of z-VAD during hypoxia and reoxygenation did not prevent Cyt *c* release in hypoxic cells, but inhibited apoptosis during reoxygenation. Adapted with permission from Saikumar et al.[10]

containing substrates results in accelerated cell death by apoptosis (Fig 1A).[10] Because apoptosis is energy dependent and reoxygenated cells had generated ATP via glycolysis, apoptotic programs had been triggered in some cells during reoxygenation (Fig 1B). It was shown that molecular oxygen was not required for this process and that oxidant mechanisms were not involved (Fig 2).[10] On the other hand, a process that led to complete loss of Cyt *c* by diffusion into the cytosol provided clear-cut evidence for mitochondrial damage during hypoxia. Cells affected in this manner underwent apoptosis, and other cells without a Cyt *c* leak recovered (Fig 1C). These findings are consistent with the discovery that Cyt *c* can trigger apoptosis by activating specific proteolytic enzymes, namely, the caspases.[22,23] Activation of caspases by Cyt *c* required an adapter protein, Apaf-1, which is a mammalian counterpart of the *C. elegans* death factor ced-4.[22,23] It also was shown that availability

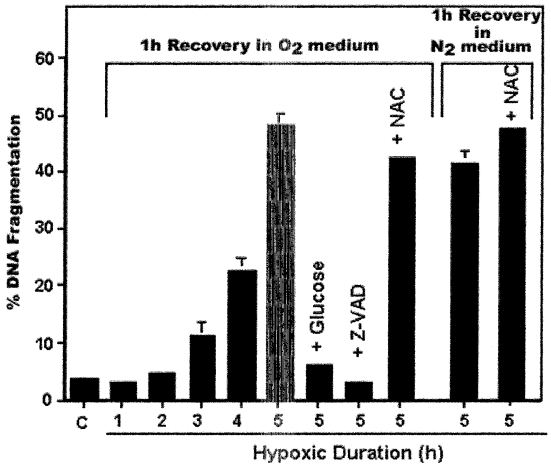

Fig 2. Role of oxygen and oxygen radicals in reoxygenation injury of rat kidney proximal tubule cells. Cell death was quantitated by measuring the fragmentation of ^{3}H-thymidine–labeled chromosomal DNA. Progressively increasing exposure to hypoxia leads to correspondingly greater fragmentation of DNA during reoxygenation (O_2 medium). Provision of glucose during hypoxia suppressed DNA fragmentation and cell death, confirming a critical role for ATP depletion in this form of cell injury. When hypoxic cells were transferred to complete growth medium pre-equilibrated with N_2/CO_2 and incubated inside the anaerobic chamber, DNA fragmentation was not inhibited (N_2 medium). Moreover, they died of apoptosis (not shown), excluding oxidative stress as the apoptotic trigger. In addition, the inclusion of an anti-oxidant, N-acetyl cysteine (NAC) during recovery under either normoxia or anoxia had no effect on cell death. Therefore, oxygen or free radicals derived from there are unlikely triggers of cell injury during reoxygenation. Data from Saikumar et al, unpublished results.

of glycolytic ATP was critical for the expression of apoptosis during reoxygenation injury because loss of Cyt *c* leads to severe impairment of mitochondrial function.[10] Thus, reoxygenated cells with Cyt *c* leaks do not enter the apoptotic program if glycolytic ATP was not available; they died by necrosis instead.[10] These considerations are particularly applicable to proximal tubule cells of the in vivo differentiated phenotype. Because energy metabolism in these cells is largely oxidative, loss of Cyt *c*, a key component of the respiratory chain, will have catastrophic consequences for energy metabolism during reoxygenation of proximal tubules, ensuring the eventuality of necrosis owing to lack of ATP. In contrast, it remains possible that regenerating proximal tubule cells and cells of the distal nephron, which are glycolytically competent, also could die by apoptosis.

MECHANISMS OF MITOCHONDRIAL CYT *C* RELEASE

The permeability transition pore (PTP) initially was considered as a pathogenetic mechanism responsible for the release of mitochondrial Cyt *c* during hypoxia or ATP depletion. PTP is a membrane pore that allows diffusion of small solutes, 1,500 daltons in size or less, across the inner mitochondrial membrane. Formation of the PTP is thought to be mediated by improper interactions between proteins in a multiprotein complex centered on the adenine nucleotide translocase, a mitochondrial cyclophilin, adenylate kinase, and porin, located in focal contacts between the inner and outer mitochondrial membranes. Although it can occur as a temporary event, the PTP rapidly can become irreversible, with resulting loss of mitochondrial homeostasis, and cause high amplitude mitochondrial swelling. Because the inner membrane has a larger surface area than the outer membrane, mitochondrial swelling can cause rupture of the outer membrane, releasing intermembrane proteins such as Cyt *c* into the cytosol. In hypoxic or ATP-depletion models, a role for PTP in Cyt *c* release has been ruled out by studies from our laboratory.[20,27] Also, in many types of apoptosis, the mitochondria remain morphologically normal, indicating a lack of mitochondrial swelling caused by the PTP. Development of the PTP in cells injured by ischemia or hypoxia has been shown in studies with organs subjected to ischemia/reperfusion, parenchymal cells isolated after hypoxia/reoxygenation, and mitochondria isolated from ischemic tissues. Although the induction and pathologic significance of the PTP has been documented by in vitro studies, extrapolation of these results to pathogenetic events during ischemic injury in vivo, or even hypoxic injury of whole isolated cells in vitro, has been fraught with difficulties. This largely has been owing to the simultaneous occurrence of several destructive processes during cell damage by ATP depletion and the lack of interventions that have sufficient power to discriminate between events that are causal, and those that evolve secondarily as effects. Thus, PTP could evolve as terminal events in cells that are compromised lethally by more specific and critical death mechanisms.

ROLE OF BCL-2 FAMILY PROTEINS IN HYPOXIC INJURY

Studies using cultured rat kidney proximal tubule cells have shown that Bax, a pro-apoptotic member of the Bcl-2 family, plays a central role in hypoxic injury of kidney cells.[20] Bcl-2 family proteins, which are divided into 2 groups based on their anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, BOO/DIVA, A1/Bfl-1, NR-13) or pro-apoptotic properties, have been shown to regulate the mitochondrial pathway leading to apoptosis.[28] Even though there is relatively low overall sequence homology, these proteins share up to 4 highly conserved regions, named Bcl-2 homology (BH) domains 1-4 (Fig 3A). The anti-apoptotic members typically contain all 4 domains, with the BH1, BH2, and BH4 domains required for their anti-apoptotic activity.[29] The pro-apoptotic members are subdivided into 2 groups: (1) multidomain proteins (Bax, Bak, Bcl-rambo, and Bok/Mtd) and (2) BH3 domain-only proteins (Bad, Bid, Bim/Bod, Bik/Nbk, Blk, Bmf, Bnip3, Hrk/DP5, Noxa, and Puma/Bbc-3). In pro-apoptotic proteins, the BH3 domain has been shown to be required for killing activity.[30] During hypoxia or ATP depletion, cytosolic Bax is translocated to mitochondria with concomitant release of mitochondrial Cyt *c* (Fig 3B). Similar release of Cyt *c* into the cytosol was detected after transient focal and global ischemia of brain,[31,32] during myocardial ischemia,[33] and in renal ischemia.[34] Increased Cyt *c* levels in the cytosol are consistent with the notion that the Bax pathway is involved in ischemic injuries. In normal cells or tissues, Bax, a multidomain protein with a transmembrane tail, is localized predominantly in the cytosol.[10,35] Bax translocation from cytosol to mitochondria has been shown after hypoxia[10] or ATP depletion,[10,36] apoptotic stimulation,[37] and cytokine withdrawal.[38] Translocation of Bax has been shown to be accompanied by conformational changes in the protein resulting in alterations of its quaternary structure. However, the identity of direct activators of Bax translocation from cytosol to mitochondria remains unknown.

Overexpression of Bcl-2, an anti-apoptotic protein, prevented hypoxic injury in kidney proximal tubule cells by inhibiting mitochondrial release of Cyt *c* (Fig 4).[10] Similarly, overexpression of Bcl-2 was shown to protect neurons of transgenic mice against ischemic insults.[39] These results have strongly suggested that the mitochondrial pathway of apoptosis is involved in the ischemic disease process. Of the 2 major pathways involved in caspase activation and apoptotic cell death,[28] the

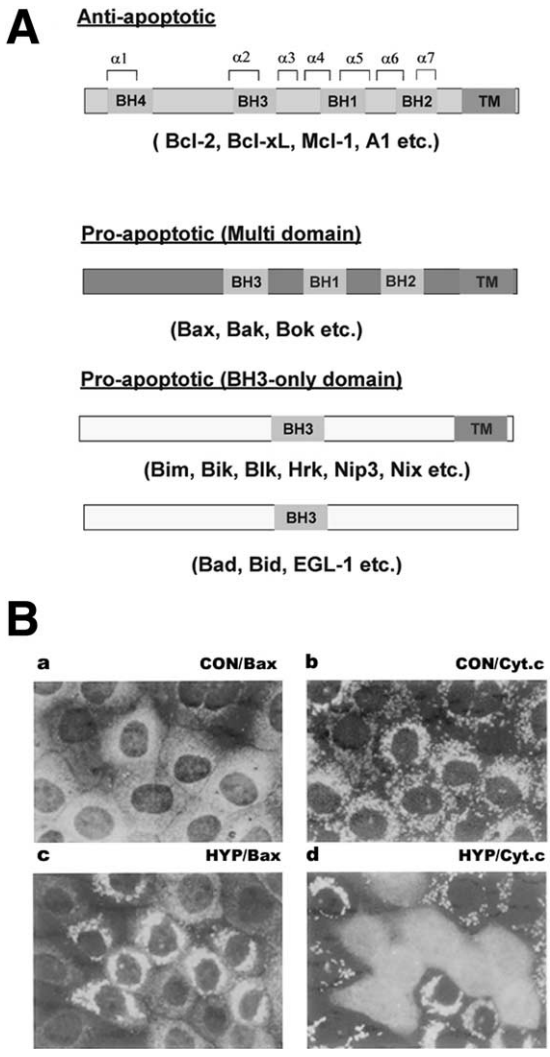

Fig 3. (A) Homologies in Bcl-2 family proteins. Bcl-2 family proteins are divided into 2 groups: anti-apoptotic and pro-apoptotic. They share Bcl-2 homology domains BH1, BH2, BH3, BH4. Some of the members also contain a transmembrane (TM) domain indicative of their membrane association. Pro-apoptotic members are subdivided into multidomain and BH3-only domain proteins. (B) Double immunolabeling of control (a, b) and hypoxic (c, d) proximal tubule cells for Bax (a, c) and Cyt *c* (b, d; Cyt *c*). In control cells, Bax is distributed diffusely in the cytoplasm (a) and Cyt *c* is present in mitochondria (b). During hypoxia, Bax is translocated to mitochondria (c) and in the same cells Cyt *c* is released into the cytosol (d). Adapted with permission from Saikumar et al.[10]

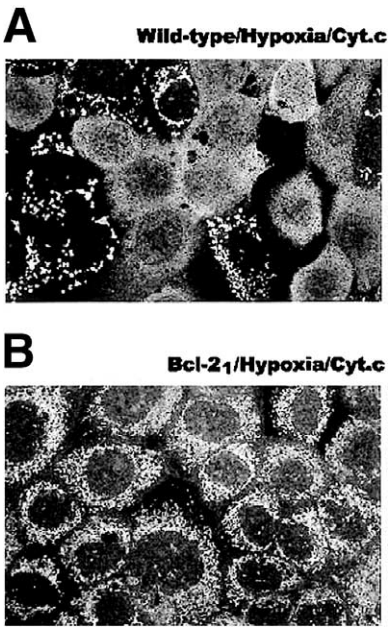

Fig 4. Effect of Bcl-2, an anti-apoptotic protein, on Cyt *c* release during hypoxia. Confocal images of hypoxic (A) wild-type and (B) Bcl-2 overexpressing (Bcl-2_1) rat kidney proximal tubule cells that are immunostained for Cyt *c*. Bcl-2 expression prevents the release of mitochondrial Cyt *c* during hypoxia. Adapted from Saikumar et al.[10]

pathway involving mitochondria plays a critical role in the induction of apoptosis by cellular stress signals, and in some cases, by death receptors. In some cell types, ligand-induced activation of death receptors such as Fas and tumor necrosis factor receptor I (TNFRI) at the cell surface is not sufficient to induce apoptosis; further amplification of the initial death receptor signal by mitochondria is required.[40] Based on these considerations, characterization of signal transduction mechanisms mediated by Bax and other pro-apoptotic Bcl-2 family proteins in mitochondria is essential to arrive at an understanding of cellular pathologies during hypoxia. Although it has been presumed that interactions between various Bcl-2 family members are involved in cell survival/cell death decisions,[41] it still is not established whether Bax activity promotes cell death and Bcl-2 inhibits Bax, or if Bcl-2 actively promotes mitochondrial viability and Bax perturbs mitochondrial integrity by inhibiting Bcl-2. Despite their opposing apoptotic activity, Bax and Bcl-2 have molecular features that have homology to the pore-forming domains of diphtheria toxin and bacterial colicins in their 3-dimensional structures,[42] and both form channels in lipid bilayers.[43,44] Interestingly, it is unclear how these proteins that form similar channels can exert opposing actions. On the other hand, studies of channel formation in synthetic lipid bilayers by members of the Bcl-2 family,[43] have suggested that pro-apoptotic members may rearrange in the outer mitochondrial membrane to allow the efflux of Cyt *c* by forming large pores rather than ion channels.

Studies using bifunctional cross-linkers have shown that translocated Bax forms homo-oligomeric structures in energy-deprived rat kidney proximal tubule cells.[36] However, translocated Bax in Bcl-2–overexpressing cells remains monomeric during ATP depletion. The protective effects of Bcl-2 did not require association between Bcl-2 and Bax proteins with a degree of proximity or affinity that is stable to conditions of immunoprecipitation or chemical cross-linking with linkers of varied spacer lengths and chemical reactivities. However, unlike in natural mitochondrial membranes, Bax and Bcl-2 readily form homodimers and heterodimers in nonionic detergents. Moreover, lack of association, observed by immunoprecipitation or by chemical cross-linking, between translocated Bax and the PTP-forming proteins such as voltage-dependent anion channel protein or the adenine nucleotide translocator protein also suggest that Bax actions are not mediated through PTP as had been suggested earlier.[45,46]

On the other hand, after Bax translocation, Bak, another proapoptotic molecule that normally resides in mitochondria, reorganizes to form homo-oligomers.[27] Bid, a BH3-only member of Bcl-2 family, whose cleavage and translocation often is involved in receptor-mediated cell death, is not involved in the oligomerization of Bax and Bak during energy deprivation. Western blotting of cross-linked Bax and Bak complexes from energy-deprived cells revealed nonoverlapping ladders consistent with the notion that Bax and Bak form separate homo-oligomeric complexes. Nevertheless, co-immunoprecipitation of Bax and Bak under these conditions suggests an affinity between homo-oligomeric complexes of Bax and Bak. Overexpression of the anti-apoptotic protein Bcl-2 in rat kidney proximal tubule cells prevented not only the oligomerization of Bax and Bak, but also the association between these 2 proteins and Cyt *c* release during energy deprivation. To identify the importance of Bax or Bak in inducing the Cyt *c*

release during energy deprivation, we have used Bax- or Bak-deficient baby mouse kidney cells. Our studies have indicated a stringent requirement of Bax for Cyt *c* release during energy deprivation. Notably, our results indicate that Bak deficiency is associated with delayed kinetics of Bax translocation but does not affect either the oligomerization of translocated Bax or the leakage of Cyt *c*. In contrast, Bax deficiency prevented both Bak homo-oligomerization and Cyt *c* release. Overall, our results[27,36] and other studies[47] support the hypothesis that Bax, through its oligomerization and stimulation of Bak homo-oligomerization, induced cell death actively by forming pores that allowed the diffusion of intermembrane proteins across outer mitochondrial membranes and that Bcl-2 blocked cell death by preventing pore formation by inhibiting the homo-oligomerization of Bax and Bak. Thus, these studies have revealed an unforeseen degree of complexity in Bcl-2 family interactions. Nevertheless, they continue to highlight a primary role for Bax in apoptosis induced by hypoxia-mediated stress signals and a major but poorly understood role for Bcl-2 as a powerful anti-apoptotic agent. Another anti-apoptotic Bcl-2 family member, Bcl-x_L, also has been shown to regulate apoptosis induced by ischemia/hypoxia in neuronal cell types.[48]

CASPASE ACTIVATION IN RENAL CELLS DURING HYPOXIA/ISCHEMIA OR ATP DEPLETION

Studies have documented the occurrence of apoptosis and activation of caspases during renal ischemia/reperfusion injury.[49-52] Because caspases are key intracellular mediators of apoptosis, their activation may play an important role in renal injury. Caspases are a family of structurally related cysteine proteases with specificity to cleave after aspartic acid residues in the P1 position of their substrates.[53] Caspases share homology and generally are synthesized as inactive precursors (zymogens). Caspase precursors are activated after internal cleavage of recognition sites by other caspases in a proteolytic cascade similar to blood clotting and complement activation. Moreover, with the assistance of adapter proteins that bring them into close proximity, caspases can process themselves, generating active enzymes in vivo.[54,55] A cascade of caspases plays the central executioner role by cleaving various mammalian death substrates that include cytosolic and nuclear proteins that have a role in DNA replication and repair, RNA splicing, cell division, and cytoskeletal structure. This biochemical carnage results in the morphologic changes that are characteristic of apoptosis. Caspases also have been classified as initiators or effectors. The upstream (initiator) caspases, unlike downstream (effector or executioner) caspases, have long prodomains containing structural motifs (eg, death effector domain [DED], or caspase recruitment domain [CARD]) that associate these enzymes to their specific activators.[54,55] At least 14 caspases encoded by distinct genes have been cloned and sequenced to date in mammals.[28] Mammalian caspases -2, -8, -9, and -10 have large prodomains; in contrast, caspase-3, -6, and -7 have short prodomains. The higher role of initiator caspases in the proteolytic cascade is supported further by the fact that initiator caspases can process themselves as well as other downstream effector caspases to generate active enzymes. In contrast, effector caspases are incapable of such initial self-activation through autocatalysis. Importantly, once the caspase cascade is initiated, the process of cell death cannot be reversed.

Caspases are expressed ubiquitously in diverse tissues including the kidney.[49,56,57] Caspases-3 and -9 were identified to be processed and activated in hypoxic cells, before reoxygenation.[58] Caspase activation was caused by ATP depletion and not by hypoxia per se. As expected, caspase activation was abolished by caspase inhibitors and overexpression of Bcl-2.[10,58] At present, there are 2 relatively well-characterized cell death pathways that result in the activation of the downstream executioner caspase-3. In hypoxic/ischemic kidneys it is possible to activate both pathways independently. One is receptor mediated and the other is mitochondrial dependent. The receptor-dependent pathway is initiated by activation of cell death receptors (Fas and tumor necrosis factor) leading to activation of procaspase-8, which in turn cleaves and activates procaspase-3. The mitochondrial-dependent pathway is triggered by released Cyt *c*. The cytosolic Cyt *c* recruits procaspase-9, Apaf-1, and dATP, and promotes caspase-9 activation. Activated caspase-9 then cleaves and activates procaspase-3 or other executioner caspases to their active form. In some cell types, these 2 separate pathways ultimately converge at the level of mitochondria to activate downstream executioner

caspases that disassemble the committed cells.[40] Activation of caspases-3, and -9 was shown to occur during hypoxia after Cyt *c* leakage, but the affected cells underwent apoptosis only during reoxygenation.[10] However, it has been difficult to delineate a hierarchy for caspase activation in these studies because processing and activation kinetics of the different caspases were indistinguishable (our unpublished results).[58] Examination of the order of activation of caspases using the selective inhibitors did not yield any clues with respect to hierarchy, suggesting a tight coupling and complexity of caspase activation.

Involvement of caspases in cell injury during hypoxia/reoxygenation has been suggested by other studies showing protective effects of caspase inhibitors.[59,60] These observations could be relevant to cell injury after ischemia/reperfusion in vivo; broad-spectrum caspase inhibitors were shown to reduce the extent of cell death during reperfusion after ischemia of the heart and brain.[61-64] However, the caspase inhibitors may not mitigate mitochondrial release of Cyt *c* during hypoxia, and therefore may not confer long-term benefits to the affected cells. Whether caspase inhibition can confer long-term viability to injured cells may indeed depend on how the caspases had been activated—by death receptor stimulation without mitochondrial involvement, or via the mitochondrial pathway.

ROLE OF INFLAMMATION IN RENAL INJURY DURING ISCHEMIA/REPERFUSION

Inflammation is considered to play a major role in ischemia/reperfusion injury as suggested by the protective effects afforded by anti-inflammatory interventions.[65] There is an increasing body of evidence to suggest that ischemia triggers the release of inflammatory mediators along with the up-regulated expression of adhesion proteins in kidney.[66] In agreement with these results, increased infiltration of neutrophils[4] and macrophages[67] into the renal tissue was seen. Although leukocytes were considered to be the main mediators of renal injury during ischemia/reperfusion,[68] the actual role of these cells still remains controversial. Administration of antibodies or antisense oligonucleotides to leukocyte adhesion molecule (CD11/CD18) or intracellular adhesion molecule-1 to rats provided both functional and morphologic protection to kidneys subjected to ischemia/reperfusion.[69-72] In addition, mice lacking the intracellular adhesion molecule-1 gene showed an impressive resistance to acute renal ischemic injury.[73] It appears that during the inflammation process, leukocytes attach strongly to the endothelium via CD11/CD18, and migration across the endothelium is regulated by the interaction between CD11/CD18 and endothelial intracellular adhesion molecule-1. After transmigration, leukocyte-mediated injury takes place. Overall, based on the observations described earlier, infiltrating cells were implicated strongly in the renal ischemia/reperfusion injury.[74] In addition to their anti-inflammatory role, some of the anti-inflammatory drugs seem to exert a hemodynamic beneficial effect on renal ischemia.[75] However, the fundamental question regarding how increased renal infiltration of leukocytes occurs had been unanswered until recently. In a recent study using a murine model of renal ischemia and reperfusion, it was shown that apoptotic inhibitors attenuated reperfusion-induced inflammation, suggesting that initial apoptosis in the parenchymal cells after ischemia/reperfusion may be one potential trigger mechanism for increased inflammation.[52] However, these studies do not rule out whether protective effects also are stemming from inhibition of apoptosis in accumulating inflammatory cells. In addition, it has been shown that reduced production of pro-inflammatory cytokines (eg, interleukin 18) by inhibiting caspase-1 activity also prevents renal ischemic injury.[76,77] By inhibiting an apoptotic chain reaction in accumulating inflammatory cells, and by inhibiting caspase-1 processing in injured tissue, caspase inhibitors may reduce inflammation, and thereby reduce the cascading parenchymal injury that is associated with inflammation.

APOPTOTIC INDUCERS AS CLINICAL TARGETS TO ALLEVIATE ISCHEMIA/REPERFUSION INJURY

Because apoptosis is responsible for increased loss of renal cells during ischemia-reperfusion injury, several strategies may be used to ameliorate renal cell death. An obvious choice will be inhibition of caspases because caspase activation is responsible for intracellular protein cleavage that results in apoptotic disintegration of cell structure. In fact, administration of caspase inhibitors markedly reduced murine renal ischemia/reperfusion injury.[52,78] However, if the caspase activation re-

quires mitochondrial Cyt *c* release, caspase inhibitors may not offer long-term protection under these circumstances owing to functional injury of mitochondria. Despite lack of protection in parenchymal cells, caspase inhibition still may be beneficial to reduce inflammation-mediated apoptosis by inhibiting chemokine production initiated by a class of inflammatory caspases (eg, caspase-1) in vascular endothelial cells or monocytes. Another potential therapeutic intervention would be the provision of growth factors that reduce apoptosis by stimulating intracellular survival pathways. In this regard, insulin-like growth factor 1, hepatocyte growth factor, and epidermal growth factor may promote cell survival during ischemia/reperfusion. In murine renal ischemia/reperfusion injury, insulin-like growth factor 1 was shown to inhibit apoptosis and inflammation as efficiently as caspase inhibitors.[52] Moreover, both epidermal growth factor and insulin-like growth factor 1 also were shown to inhibit apoptosis and ameliorate renal injury in other experimental models such as obstructive nephropathy and toxic nephropathy.[79-81]

Another report suggested that pretreatment with guanosine ameliorates ischemic acute renal failure in a murine model and this improvement is associated with decreased apoptosis in renal cells.[82] The protective effects of guanosine during reperfusion were suggested to be mediated through inhibition of apoptosis. Pifithrin-α, a specific inhibitor of p53, also can mimic the protective effects of guanosine, most probably guanosine and pifithrin-α protect by inhibiting p53-mediated transcriptional activation of downstream targets such as p21 and Bax.[83]

Infiltrating leukocytes may exacerbate renal injury by inducing renal cell apoptosis by releasing tumor necrosis factor α, Fas ligand, and nitric oxide by macrophages or reactive oxygen species and degradative enzymes by neutrophils. Therefore, therapeutic interventions that reduce or inhibit leukocyte infiltration and/or toxic cytokine action also would help to ameliorate ischemia/reperfusion injury in kidney. In view of the considerations described earlier, it seems likely that renal damage during hypoxia/ischemia involves apoptosis in either parenchymal or inflammatory cells or both. Therefore, further research that considers multiple targets will be beneficial in obtaining effective treatment modalities.

REFERENCES

1. Wirthensohn G, Guder WG: Renal substrate metabolism. Physiol Rev 66:469-497, 1986

2. Nicotera P, Leist M, Ferrando-May E: Intracellular ATP, a switch in the decision between apoptosis and necrosis. Toxicol Lett 102-103:139-142, 1998

3. Daemen MA, van de Ven MW, Heineman E, et al: Involvement of endogenous interleukin-10 and tumor necrosis factor-alpha in renal ischemia-reperfusion injury. Transplantation 67:792-800, 1999

4. De Greef KE, Ysebaert DK, Ghielli M, et al: Neutrophils and acute ischemia-reperfusion injury. J Nephrol 11:110-122, 1998

5. Weinberg JM, Venkatachalam MA, Roeser NF, et al: Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. Proc Natl Acad Sci U S A 97:2826-2831, 2000

6. Weinberg JM, Venkatachalam MA, Roeser NF, et al: Anaerobic and aerobic pathways for salvage of proximal tubules from hypoxia-induced mitochondrial injury. Am J Physiol 279:F927-F943, 2000

7. Weinberg JM, Venkatachalam MA, Roeser NF, et al: Energetic determinants of tyrosine phosphorylation of focal adhesion proteins during hypoxia/reoxygenation of kidney proximal tubules. Am J Pathol 158:2153-2164, 2001

8. Borutaite V, Morkuniene R, Budriunaite A, et al: Kinetic analysis of changes in activity of heart mitochondrial oxidative phosphorylation system induced by ischemia. J Mol Cell Cardiol 28:2195-2201, 1996

9. Wyllie AH: Death from inside out: An overview. Philos Trans R Soc Lond B Biol Sci 345:237-241, 1994

10. Saikumar P, Dong Z, Patel Y, et al: Role of hypoxia-induced Bax translocation and cytochrome c release in reoxygenation injury. Oncogene 17:3401-3415, 1998

11. Lieberthal W, Levine JS: Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. Am J Physiol 271:F477-F488, 1996

12. Kajstura J, Liu Y, Baldini A, et al: Coronary artery constriction in rats: Necrotic and apoptotic myocyte death. Am J Cardiol 82:30K-41K, 1998

13. Li Y, Powers C, Jiang N, et al: Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. J Neurol Sci 156:119-132, 1998

14. Noda T, Iwakiri R, Fujimoto K, et al: Programmed cell death induced by ischemia-reperfusion in rat intestinal mucosa. Am J Physiol 274:G270-G276, 1998

15. Wiegele G, Brandis M, Zimmerhackl LB: Apoptosis and necrosis during ischaemia in renal tubular cells (LLC-PK1 and MDCK). Nephrol Dial Transplant 13:1158-1167, 1998

16. Bonfoco E, Krainc D, Ankarcrona M, et al: Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 92:7162-7166, 1995

17. Leist M, Single B, Castoldi AF, et al: Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 185:1481-1486, 1997

18. Leist M, Nicotera P: The shape of cell death. Biochem Biophys Res Commun 236:1-9, 1997

19. McCarthy NJ, Whyte MK, Gilbert CS, et al: Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J Cell Biol 136:215-227, 1997

20. Saikumar P, Dong Z, Weinberg JM, et al: Mechanisms of cell death in hypoxia/reoxygenation injury. Oncogene 17: 3341-3349, 1998

21. Ashkenazi A, Dixit VM: Death receptors: Signaling and modulation. Science 281:1305-1308, 1998

22. Li P, Nijhawan D, Budihardjo I, et al: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489, 1997

23. Zou H, Henzel WJ, Liu X, et al: Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405-413, 1997

24. Xiang J, Chao DT, Korsmeyer SJ: BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. Proc Natl Acad Sci U S A 93:14559-14563, 1996

25. Miller TM, Moulder KL, Knudson CM, et al: Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol 139:205-217, 1997

26. Wilson PD: Use of cultured renal tubular cells in the study of cell injury. Miner Electrolyte Metab 12:71-83, 1986

27. Mikhailov V, Mikhailova M, Degenhardt K, et al: Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release. J Biol Chem 25:25, 2002

28. Saikumar P, Dong Z, Mikhailov V, et al: Apoptosis: Definition, mechanisms, and relevance to disease. Am J Med 107:489-506, 1999

29. Reed JC, Zha H, Aime-Sempe C, et al: Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 406:99-112, 1996

30. Bouillet P, Strasser A: BH3-only proteins—evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J Cell Sci 115:1567-1574, 2002

31. Fujimura M, Morita-Fujimura Y, Murakami K, et al: Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab 18:1239-1247, 1998

32. Antonawich FJ: Translocation of cytochrome c following transient global ischemia in the gerbil. Neurosci Lett 274: 123-126, 1999

33. Borutaite V, Budriunaite A, Morkuniene R, et al: Release of mitochondrial cytochrome c and activation of cytosolic caspases induced by myocardial ischaemia. Biochim Biophys Acta 1537:101-109, 2001

34. Benitez-Bribiesca L, Gomez-Camarillo M, Castellanos-Juarez E, et al: Morphologic, biochemical and molecular mitochondrial changes during reperfusion phase following brief renal ischemia. Ann N Y Acad Sci 926:165-179, 2000

35. Hsu YT, Youle RJ: Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J Biol Chem 273:10777-10783, 1998

36. Mikhailov V, Mikhailova M, Pulkrabek DJ, et al: Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J Biol Chem 276:18361-18374, 2001

37. Hsu YT, Wolter KG, Youle RJ: Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A 94:3668-3672, 1997

38. Goping IS, Gross A, Lavoie JN, et al: Regulated targeting of BAX to mitochondria. J Cell Biol 143:207-215, 1998

39. Martinou JC, Dubois-Dauphin M, Staple JK, et al: Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017-1030, 1994

40. Scaffidi C, Schmitz I, Zha J, et al: Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. J Biol Chem 274:22532-22538, 1999

41. Korsmeyer SJ, Shutter JR, Veis DJ, et al: Bcl-2/Bax: A rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol 4:327-332, 1993

42. Muchmore SW, Sattler M, Liang H, et al: X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature 381:335-341, 1996

43. Schlesinger PH, Gross A, Yin XM, et al: Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc Natl Acad Sci U S A 94:11357-11362, 1997

44. Minn AJ, Velez P, Schendel SL, et al: Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature 385:353-357, 1997

45. Marzo I, Brenner C, Zamzami N, et al: Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science 281:2027-2031, 1998

46. Shimizu S, Narita M, Tsujimoto Y: Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483-487, 1999

47. Antonsson B, Montessuit S, Lauper S, et al: Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem J 345:271-278, 2000

48. Parsadanian AS, Cheng Y, Keller-Peck CR, et al: Bcl-xL is an antiapoptotic regulator for postnatal CNS neurons. J Neurosci 18:1009-1019, 1998

49. Kaushal GP, Singh AB, Shah SV: Identification of gene family of caspases in rat kidney and altered expression in ischemia-reperfusion injury. Am J Physiol 274:F587-F595, 1998

50. Feldenberg LR, Thevananther S, del Rio M, et al: Partial ATP depletion induces Fas- and caspase-mediated apoptosis in MDCK cells. Am J Physiol 276:F837-F846, 1999

51. Chien CT, Hsu SM, Chen CF, et al: Prolonged ischemia potentiates apoptosis formation during reperfusion by increase of caspase 3 activity and free radical generation. Transplant Proc 32:2065-2066, 2000

52. Daemen MA, van 't Veer C, Denecker G, et al: Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. J Clin Invest 104:541-549, 1999

53. Thornberry NA, Lazebnik Y: Caspases: Enemies within. Science 281:1312-1316, 1998

54. Muzio M, Stockwell BR, Stennicke HR, et al: An induced proximity model for caspase-8 activation. J Biol Chem 273:2926-2930, 1998

55. Salvesen GS, Dixit VM: Caspase activation: The induced-proximity model. Proc Natl Acad Sci U S A 96:10964-10967, 1999

56. Ali SM, Wong VY, Kikly K, et al: Apoptosis in poly-

cystic kidney disease: Involvement of caspases. Am J Physiol 278:R763-R769, 2000

57. Truong LD, Choi YJ, Tsao CC, et al: Renal cell apoptosis in chronic obstructive uropathy: The roles of caspases. Kidney Int 60:924-934, 2001

58. Dong Z, Saikumar P, Patel Y, et al: Serine protease inhibitors suppress cytochrome c-mediated caspase-9 activation and apoptosis during hypoxia-reoxygenation. Biochem J 347: 669-677, 2000

59. Mold C, Morris CA: Complement activation by apoptotic endothelial cells following hypoxia/reoxygenation. Immunology 102:359-364, 2001

60. Kovacs P, Bak I, Szendrei L, et al: Non-specific caspase inhibition reduces infarct size and improves post-ischaemic recovery in isolated ischaemic/reperfused rat hearts. Naunyn Schmiedebergs Arch Pharmacol 364:501-507, 2001

61. Huang JQ, Radinovic S, Rezaiefar P, et al: In vivo myocardial infarct size reduction by a caspase inhibitor administered after the onset of ischemia. Eur J Pharmacol 402:139-142, 2000

62. Katz LM, Lotocki G, Wang Y, et al: Regulation of caspases and XIAP in the brain after asphyxial cardiac arrest in rats. Neuroreport 12:3751-3754, 2001

63. Hara H, Friedlander RM, Gagliardini V, et al: Inhibition of interleukin 1 beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci U S A 94:2007-2012, 1997

64. Seyfried DM, Veyna R, Han Y, et al: A selective cysteine protease inhibitor is non-toxic and cerebroprotective in rats undergoing transient middle cerebral artery ischemia. Brain Res 901:94-101, 2001

65. Brady HR: Leukocyte adhesion molecules: Potential targets for therapeutic intervention in kidney diseases. Curr Opin Nephrol Hypertens 2:171-182, 1993

66. Sheridan AM, Bonventre JV: Cell biology and molecular mechanisms of injury in ischemic acute renal failure. Curr Opin Nephrol Hypertens 9:427-434, 2000

67. Rabb H, Daniels F, O'Donnell M, et al: Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. Am J Physiol 279:F525-F531, 2000

68. Rabb H, O'Meara YM, Maderna P, et al: Leukocytes, cell adhesion molecules and ischemic acute renal failure. Kidney Int 51:1463-1468, 1997

69. Rabb H, Mendiola CC, Dietz J, et al: Role of CD11a and CD11b in ischemic acute renal failure in rats. Am J Physiol 267:F1052-F1058, 1994

70. Kelly KJ, Williams WW Jr, Colvin RB, et al: Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. Proc Natl Acad Sci U S A 91:812-816, 1994

71. Haller H, Dragun D, Miethke A, et al: Antisense oligonucleotides for ICAM-1 attenuate reperfusion injury and renal failure in the rat. Kidney Int 50:473-480, 1996

72. Haug CE, Colvin RB, Delmonico FL, et al: A phase I trial of immunosuppression with anti-ICAM-1 (CD54) mAb in renal allograft recipients. Transplantation 55:766-773, 1993

73. Kelly KJ, Williams WW Jr, Colvin RB, et al: Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. J Clin Invest 97:1056-1063, 1996

74. Lauriat S, Linas SL: The role of neutrophils in acute renal failure. Semin Nephrol 18:498-504, 1998

75. Ventura CG, Coimbra TM, de Campos SB, et al: Mycophenolate mofetil attenuates renal ischemia/reperfusion injury. J Am Soc Nephrol 13:2524-2533, 2002

76. Melnikov VY, Ecder T, Fantuzzi G, et al: Impaired IL-18 processing protects caspase-1-deficient mice from ischemic acute renal failure. J Clin Invest 107:1145-1152, 2001

77. Melnikov VY, Faubel S, Siegmund B, et al: Neutrophil-independent mechanisms of caspase-1- and IL-18-mediated ischemic acute tubular necrosis in mice. J Clin Invest 110:1083-1091, 2002

78. Daemen MA, de Vries B, Buurman WA: Apoptosis and inflammation in renal reperfusion injury. Transplantation 73: 1693-1700, 2002

79. Chevalier RL, Goyal S, Wolstenholme JT, et al: Obstructive nephropathy in the neonatal rat is attenuated by epidermal growth factor. Kidney Int 54:38-47, 1998

80. Chevalier RL, Goyal S, Kim A, et al: Renal tubulointerstitial injury from ureteral obstruction in the neonatal rat is attenuated by IGF-1. Kidney Int 57:882-890, 2000

81. Klahr S: Urinary tract obstruction. Semin Nephrol 21: 133-145, 2001

82. Kelly KJ, Plotkin Z, Dagher PC: Guanosine supplementation reduces apoptosis and protects renal function in the setting of ischemic injury. J Clin Invest 108:1291-1298, 2001

83. Kelly KJ, Plotkin Z, Vulgamott SL, et al: P53 mediates the apoptotic response to gtp depletion after renal ischemia-reperfusion: Protective role of a p53 inhibitor. J Am Soc Nephrol 14:128-138, 2003