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Mechanisms of ischemic acute renal failure

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Case presentation

A 26-year-old white man suffered a gunshot wound to the right groin. He lost a large amount of blood and had a cardiopulmonary arrest. After intubation, CPR was administered, and he was brought to the hospital, where he was found to have a right common femoral artery and vein transection. The femoral vein and external iliac artery were ligated, and a graft was placed to repair the femoral artery. Postoperatively he continued to lose blood and became hypotensive and oliguric. He received 10 units of packed red blood cells and 2 units of fresh frozen plasma. On transfer to the Massachusetts General Hospital, his blood pressure was 45/20 mm Hg. A second surgical procedure disclosed a large hematoma and diffuse bleeding as well as a retroperitoneal hematoma; hydronephrosis was not found, and the bladder and ureteral system were patent. The blood urea nitrogen (BUN) on admission was 21 mg/dl and the serum creatinine was 2.3 mg/dl. Although he received another 21 units of packed red blood cells, 20 units of fresh frozen plasma, and 10 units of platelets, he remained hypotensive; phenylephrine and norepinephrine maintained his blood pressure at 105/62 mm Hg. Furosemide and mannitol were administered by continuous infusion. His urine output decreased to 30–40 ml/hr, and examination of the urine revealed many granular pigmented casts. On the third hospital day, the BUN and serum creatinine were 44 mg/dl and 4.8 mg/dl respectively; he continued to require pressors for blood pressure support.

On the 5th hospital day, his urine output increased from 70 to 100 ml/hr. On the 6th hospital day, the BUN and serum creatinine peaked at 91 mg/dl and 7.2 mg/dl respectively. On the 8th hospital day, the serum creatinine was 4.9 mg/dl and urine output remained greater than one liter/day. On day 13, the BUN was 78 mg/dl and the serum creatinine was 3.5 mg/dl. A blood culture grew methicillin-resistant *Staphylococcus aureus*. Recurrent episodes of bradycardia required placement of a cardiac pacemaker; his blood pressure decreased from 130/80 mm Hg to 100/60 mm Hg. The serum albumin was 1.5 g/dl. The BUN and serum creatinine began to increase again, although he remained nonoliguric with a urine output greater than one liter/day. Urinalysis revealed numerous granular casts and epithelial cells. Urine osmolality was 370 mOsm/liter. The urine contained no white blood cells and an insignificant amount of protein. The BUN and serum creatinine increased to 154 mg/dl and 7.4 mg/dl respectively on the 17th hospital day, and peritoneal dialysis was initiated. The urine output decreased to 10 ml/hr. Peritoneal dialysis was continued until the 36th hospital day. On the 43rd hospital day, the BUN was 28 mg/dl and the serum creatinine was 1.2 mg/dl. The hospital course was complicated by anoxic encephalopathy, recurrent sepsis, and respiratory failure. The patient was discharged to his home two months later with a BUN of 10 mg/dl and a serum creatinine of 1.1 mg/dl.

Discussion

DR. JOSEPH V. BONVENTRE (*Medical Services, Massachusetts General Hospital; Associate Professor of Medicine, Harvard Medical School; and Associate Director of The Harvard/Massachusetts Institute of Technology Division of Health Sciences and Technology, Boston, Massachusetts*): This patient had two episodes of acute renal failure early in his hospitalization after a nearly fatal gunshot wound. The first episode, clearly caused by blood loss, resulted in hypotension and renal ischemia. Ischemia also contributed to the second episode, but his medical condition was then complicated by sepsis and a low serum albumin, which likely predisposed him to acute ischemic renal failure. In this Forum, I will review the pathophysiologic mediators that have been implicated in the development of ischemic acute renal failure. I will limit my discussion to ischemia and its ramifications and will not discuss sepsis, nephrotoxins, or other aspects of multisystem disease that frequently contribute to the development of ischemic acute renal failure.

The pathophysiology of ischemic acute renal failure is complex. By definition, this form of acute renal failure is initiated by inadequate blood flow. Inadequate blood flow can be due to decreased cardiac output, as can occur with acute cardiac decompensation. It can accompany renal artery stenosis or occlusion, or it can be due to intrarenal smaller vessel lesions such as atherosclerosis, atheroemboli, or vasculitis. It has been known for many years that restoration of total renal blood flow to normal shortly after an ischemic insult does not prevent the maintenance phase of acute renal failure. Thus, while reduced renal blood flow and/or alterations in the distribution of renal blood flow may contribute to the maintenance phase of acute

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renal failure, ischemia sets off a sequence of epithelial cell pathophysiologic processes that, once initiated, perpetuates the tissue damage and functional defects independent of total renal blood flow.

Abnormal regulation of local blood flow may play an important role in ischemic acute renal failure. The functional integrity of the microvasculature depends on the proper balance between vasoconstrictive and vasodilatory factors. Damage to the endothelium or alteration in endothelial function can result in local vasoconstriction due to increased production of vasoconstrictive substances such as endothelin and to decreased production of vasodilatory substances such as nitric oxide. We have found, for example, that the vasodilatory response to acetylcholine decreases in aortic rings exposed to low oxygen tension [1]. Alterations in endothelial cell function can be important in the local loss of autoregulation that occurs in ischemic renal failure [2].

There is evidence that heterogeneity of blood flow plays an important role in the pathophysiology of ischemic renal failure. The early pathologic descriptions of "lower nephron nephrosis" by Oliver emphasized the patchy nature of the tubular injury and the localization of damage to the distal part of the nephron [3]. This could be due to differential susceptibility of nephron segments to ischemia and/or to differential levels of ischemia due to heterogeneity of blood flow. A pale cortex and "hyperemic" medulla generally have been considered features of acute ischemic renal failure [4]. Some investigators have interpreted these findings as evidence for redistribution of blood flow from cortex to medulla; others believe these features point to increased congestion in the medulla. Vascular congestion in the outer medulla [5] and decreased blood flow to this region [6] occur in experimental ischemic acute renal failure in rats. Hellberg et al measured the distribution of blood flow in the rat kidney after 60 minutes of ischemia using single-fiber laser Doppler probes placed in superficial cortex, outer medulla, and inner medulla [7]. After ischemia, blood flow decreased to 60% of pre-ischemic levels in superficial cortex and to 16% of pre-ischemic levels in outer medulla, but increased to 125% of control values in inner medulla. This decrease in blood flow to the outer medulla diminishes the oxygen and nutrient delivery to the tubules in this region, thus increasing the risk of cell injury. Swelling of endothelial or tubular epithelial cells, especially in the outer medulla, may be responsible for interference with flow through the vasa recta and for compromise of the blood flow. Thus, maneuvers that maintain endothelial cell integrity, dilate the vasculature, or prevent tubular cell swelling may be advantageous in preserving renal function.

The vulnerability of the tubules in the outer medulla is also increased by the vasa recta countercurrent exchange of oxygen that results in a marked drop-off in oxygen tension with increasing distance into the medulla from the cortex [8]. Many medullary cells increase their rates of glycolysis in an anaerobic environment, but the thick ascending limb cells in the outer medulla cannot preserve their ATP levels despite enhanced glycolysis [9]. Epstein, Brezis, and Rosen, using the isolated perfused kidney preparation, have emphasized the vulnerability of the thick ascending limb as reflected by structural damage to this nephron segment. They attribute this vulnerability to the mismatch between the vigorous metabolic demand of, and the inadequate oxygen delivery to, the thick ascending limb [10–12].

Tubular cell damage

Generalized and/or localized ischemia to renal tissue damages the tubular cells themselves. Injured tubular cells release "cellular debris" into the tubular lumen, and this process contributes to cast formation [13], tubular lumen obstruction, increased tubular pressures [14, 15], and a marked reduction in single-nephron glomerular filtration rate. The damaged cells slough, leaving a denuded basement membrane; increased tubular fluid then leaks back into the peritubular capillaries. The resulting effective reduction in GFR is likely much more important than are glomerular endothelial and epithelial cell changes and the subsequent effects on the glomerular capillary ultrafiltration coefficient [16, 17]. Furthermore, the tubular cell swelling in the confined space of the outer medulla mechanically adds to capillary obstruction and causes medullary congestion, decreased medullary blood flow, and further ischemia and tubular cell injury. These changes establish a positive-feedback process that exacerbates the injury. The damage to the tubular cell represents the final event that accounts for much of the pathophysiology of ischemic acute renal failure.

In this discussion I will review the many factors that are believed to contribute to tubular cell injury and death in ischemic acute renal failure. Many episodes of human acute ischemic renal failure are multifactorial; affected patients are frequently septic, are receiving nephrotoxic drugs such as aminoglycosides, have multiple organ system failure, and have suboptimal nutritional status [18]. I will, however, limit my discussion to ischemia alone, as the renal effects of other factors contributing to acute ischemic renal failure easily could be topics for many Nephrology Forums. Furthermore, since this series recently contained a review by Toback of the factors potentially operative in repair after acute renal failure [19], I will not deal with that aspect of acute ischemic renal failure, although I believe that understanding the repair process is just as important as understanding the features of the injury and cell death itself. Time and space will limit my discussion to an overview of the controversies that mark this field. Some of the disagreements regarding interpretation of data relate to the use of multiple experimental "in-vivo" models and the inability to exactly mimic human acute renal failure in an experimental model. Because of the difficulty in studying cellular function and biochemistry in vivo, many of the studies have been performed on isolated tubules in vitro under conditions designed to mimic those in vivo, but clearly these are not able to reproduce all the features of ischemia, which include: hypoxia, substrate depletion, metabolic product accumulation, decreases in pH, and extracellular electrolyte abnormalities.

The mechanisms responsible for the tubular epithelial cell injury and death that occur as a result of ischemia are controversial [20, 21]. It is clear that many cellular systems change markedly with ischemia. No general agreement exists, however, as to which of these metabolic changes cause cell death and which are secondary to the cellular injury. Nevertheless we should consider each of these proposed pathophysiologic mechanisms because our attempts at protecting the cells ultimately will depend on an understanding of which factors are primary. First I will briefly discuss the morphologic correlates of cell

injury that occur during the early stages of acute renal failure prior to cell regeneration and repair. I then will discuss various pathophysiologic mechanisms of cell injury including the roles of adenine nucleotides, reactive oxygen species, acidosis, calcium, phospholipases, amino acids, and proteases. Finally I will evaluate a potential role for apoptosis in the pathophysiology of the tissue injury.

Morphologic correlates of ischemic tubular cell injury. Morphologic changes that occur with ischemic acute renal failure provide some insight into its pathophysiology, although frequently there is not a good correlation between structural and functional changes in human acute ischemic renal failure. The earliest morphologic changes that occur with ischemia include loss of the apical brush border and blebbing of apical membranes. Molitoris and colleagues found that after 10 minutes of ischemia, sodium-potassium ATPase activity, normally present only on the basolateral aspects of proximal tubular cells, is found on the apical membrane also [22–25]. In addition, apical membrane lipid composition changes markedly after periods of ischemia as short as 5 minutes. These changes, together with the loss of basolateral interdigitations [26], likely account for the functional decrease in sodium and sodium-coupled proximal transport properties. Changes in the actin cytoskeleton might be responsible for the loss of polarity of the proximal tubule cell [27].

With more advanced ischemic injury, vacuoles form within the cell, mitochondria swell, "flocculent mitochondrial densities" appear, and nuclei undergo pyknosis. Cells detach from the basement membrane, leaving gaps, and the remaining cells "flatten-out" along the basement membrane. Cellular debris and intratubular protein form casts and obstruct the tubules, causing increased pressures proximal to the obstruction; the fluid that leaks out from the obstructed tubules causes tissue edema. Later I will discuss the possibility that another form of injury, so-called "apoptosis," contributes to cell death in ischemic acute renal failure.

Cell swelling represents an important response to ischemia. Cell volume is controlled by the sodium-potassium ATPase pump, which produces a negative intracellular charge and a low intracellular sodium concentration [28, 29]. With oxygen deprivation and ATP depletion, the sodium-potassium ATPase pump becomes inactive; the cells thus become depolarized, and intracellular sodium and chloride accumulate. Using electron microprobe techniques, Mason and colleagues measured total cellular sodium and chloride in proximal and distal tubules after ischemia in rats [30]. Sodium and chloride content increased both in proximal and distal cells, with the greater changes in proximal cells. As a result, the cells take up water and swell.

An intact cytoskeleton appears to help maintain normal proximal cell volume [31]. Disruption of the cytoskeleton as a consequence of ischemia can contribute to cell swelling. The addition of impermeant solutes to renal preservation solutions in kidneys destined for transplantation prevents this cell swelling and helps preserve kidney viability [32].

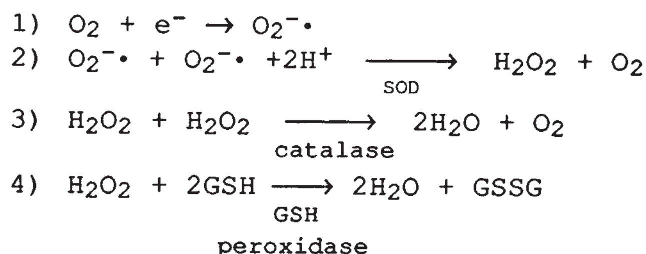
Cell swelling contributes to obstruction of the tubular lumen and compromises blood supply to regions of the kidney precariously dependent on delicate vasa recta to provide oxygen delivery. Although a reduction in cell swelling does not prevent ischemic cell death in an isolated cell preparation, in the organ, prevention of cell swelling does prevent tubular and venous obstruction and allows more oxygen delivery to the partially ischemic or post-ischemic tissue. Agents such as mannitol that inhibit cell swelling partially protect the kidney against the functional deficits induced by ischemia and also decrease outer medullary congestion [5, 33].

Adenine nucleotide metabolism. When a cell or tissue that functions primarily on aerobic metabolism becomes ischemic, cellular ATP levels, the energy currency of the cell, fall rapidly. In the kidney, complete ischemia for 10 minutes reduces ATP levels 70% to 90% [34]. Cortical levels drop much more dramatically than do medullary levels [35, 36], likely because of the glycolytic metabolism of some medullary cells, which are adapted to an environment that is relatively anoxic even under normal conditions. In the outer and inner medullary collecting ducts, glycolytic metabolism alone can maintain ATP levels at 60% to 70% of normal [9, 37, 38] and glycolysis alone can sustain acid secretion in the outer medullary collecting duct [38], the latter being a dominant function of this nephron segment. Cells in these nephron segments also can increase their glycolytic rate more efficiently when oxidative metabolism is blocked than can the proximal tubule cells [39]. Increased medullary glycolysis does not mean, however, that all medullary cells are insensitive to an ischemic insult. Given the marked heterogeneity of cellular structure within this region, an aggregate measurement of glycolytic rates or ATP levels in the medulla does not always reflect levels within some specific cells. The medullary thick ascending limb, for example, cannot sustain normal ATP levels in the presence of inhibition of oxidative metabolism despite its increased glycolytic rate [9].

A plethora of cellular processes critically depend on hydrolysis of the high energy phosphate of ATP. These processes, which include protein synthesis, lipogenesis, and membrane transport, cease or become significantly impaired when cellular ATP is markedly depleted. Ion gradients dissipate without the ATP necessary for the function of the corresponding ion transporter ATPases. Sodium and calcium accumulate in the cell. Deacylation-reacylation cycling is disrupted, and fatty acids accumulate because not enough energy is available for reacylation. Acidosis develops as the cell derives more energy from glycolytic metabolism.

With ischemia, ATP is degraded to ADP and AMP. The AMP is then acted upon by 5′-nucleotidase (or is first converted to IMP by adenylate deaminase prior to the action of 5′-nucleotidase) with the resultant formation of adenosine, inosine, and hypoxanthine. The nucleotides (ATP, ADP, AMP, IMP) are relatively impermeable to cells; in contrast, the nucleosides (adenosine and inosine) and the base (hypoxanthine) can leak out of cells and decrease the purine substrate pool. Furthermore, the hypoxanthine can be converted by xanthine oxidase to uric acid. In the process, reactive oxygen species are formed, and these can contribute to the ischemic damage.

Not all the cellular injury that occurs with ischemia happens during the ischemic period. A significant fraction of the injury occurs after reperfusion. During reoxygenation, the efficiency of ATP reaccumulation is critically important for cell survival. During reperfusion and reoxygenation, the possible pathways for resynthesis of ATP are de-novo purine synthesis from nonpurine precursors, and "salvage pathways" from AMP, adenosine, adenine, or other purine bases, nucleosides, or



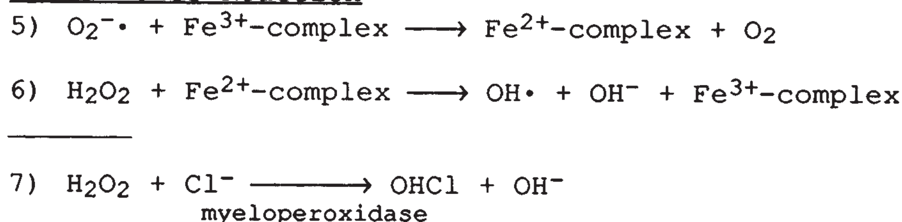


Fig. 1. *Reactions involved in metabolism of molecular oxygen.*

nucleotides. De-novo synthesis probably plays little role in the recovery of ATP levels after ischemia.

The importance of depletion of the adenine nucleotide pool has been evaluated in experiments designed to enhance this pool in an attempt to protect the kidney against ischemic injury. Siegel and colleagues, in a large series of experiments, demonstrated that intravenous infusion of ATP and magnesium chloride protected rat kidneys against 30 minutes of ischemia, even when the agents were administered after the ischemic insult [40, 41]. This protection correlated with an increased rate of recovery of ATP levels [42]. Exogenous AMP was as effective as ATP in enhancing the recovery rate of tissue ATP levels upon reperfusion; this finding led to the conclusion that the administration of adenine nucleotides enhances the nucleotide precursor pool available for the resynthesis of ATP. Further evidence for the importance of maintenance of the purine pool for restoration of ATP levels derives from experiments performed with the ADP analogue, adenosine α,β-methylene diphosphate, which inhibits 5′-nucleotidase [43]. Pretreatment with this agent increased the residual nucleotide pool after 45 minutes of ischemia and protected kidneys against ischemic injury as determined by inulin clearance 24 hours after the insult.

Reactive oxygen species. Reactive oxygen species (ROS) have been implicated in the renal cell injury that occurs with reperfusion after ischemia. Three lines of evidence have been presented to suggest that ROS are involved. (*1*) Products of lipid peroxidation are generated on reperfusion, and these are presumed to derive from ROS action on membrane lipids [44, 45]. (*2*) Scavengers such as superoxide dismutase (SOD), glutathione, and vitamin E, as well as inhibitors of ROS production, such as the iron chelator deferoxamine, have been reported to protect against ischemic injury [46, 47]. (*3*) Exposure of kidney subcellular organelles or microsomes to ROS-generating systems mimics some features of ischemic injury [45, 48].

In mammals, the chemical reduction of oxygen can occur via the mitochondrial tetravalent reduction process or can result from a series of sequential univalent reductions (Fig. 1). The first univalent reduction yields superoxide ($O_2^-\cdot$); two superoxide radicals then react to generate hydrogen peroxide and molecular oxygen in a reaction markedly potentiated by superoxide dismutase. Hydrogen peroxide can (*1*) be converted to molecular oxygen and water by catalase; (2) oxidize glutathione (GSH) to GSSH, a reaction catalyzed by GSH peroxidase; (*3*) participate with superoxide and Fe^{2+}/Fe^{3+} in a two-step "Haber-Weiss" reaction that generates the hydroxyl radical; (*4*) react with iron to generate highly reactive ferryl or perferryl Fe-O· complexes; or (*5*) react with halides in the presence of the neutrophil enzyme myeloperoxidase to generate hypohalous acids that also are very reactive.

Reactive oxygen species may derive from a variety of sources in post-ischemic tissue. When mitochondria are deprived of oxygen, the electron transport chain intermediates become more reduced and, when oxygen delivery to the tissue is restored, free electrons can increase superoxide generation. A second important source of ROS is xanthine oxidase. In ischemic tissue, xanthine oxidase levels increase due to conversion from xanthine dehydrogenase. Under normoxic conditions, xanthine dehydrogenase catalyzes the transfer of electrons to nicotine adenine dinucleotide (NAD) and oxidizes xanthine and hypoxanthine to uric acid. With ischemia and reperfusion, however, xanthine dehydrogenase is converted to xanthine oxidase by a Ca^{2+}-dependent protease [49]. Xanthine oxidase uses molecular oxygen as an electron acceptor and generates superoxide during the oxidation of hypoxanthine, which increases in concentration in the post-ischemic tissue. In one study, tissue hypoxanthine levels increased approximately 300-fold after 60 minutes of renal ischemia [50]. This source of ROS is likely more relevant in rats than in humans; the human kidney has low levels of xanthine oxidase [51].

Prostaglandin H (PGH) synthase and lipoxygenase, the enzymes involved in the metabolism of arachidonic acid, comprise a third source of ROS. Both of these enzymes produce superoxide in the presence of NADH or NADPH [52]. The neutrophils present in the post-ischemic tissue are a fourth source of ROS. These neutrophils can directly participate in the damage to the epithelial or endothelial cell, or they can act via the production of cytokines, such as leukotrienes, which can

further compromise the local blood supply by causing vasoconstriction [53, 54]. Activated neutrophils produce superoxide as a result of activity of the NADPH oxidase enzyme [55]. A fifth source, the auto-oxidation of catecholamines, generates superoxide and hydrogen peroxide [56, 57]. Finally, the oxidative enzymes of the endoplasmic reticulum, such as those of the P450 mixed-function oxidase system, and the peroxisomes represent other potential sources of ROS.

Reactive oxygen species can damage tissue in a variety of ways. They can cause lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid of membrane phospholipids; a conjugated diene forms after molecular rearrangement of the fatty acid. The diene then reacts with oxygen to form a peroxide radical, which can remove hydrogen atoms from other lipids, generating a chain reaction. Lipid peroxidation can increase plasma and subcellular membranes' permeability [58], impair enzymatic processes and ion pumps [45], and damage DNA [59, 60]. In addition, direct oxidation of membrane proteins occurs [61], affecting critical proteins such as the sodium-potassium ATPase and the Ca^{2+} ATPase.

The role of ROS in ischemic renal injury remains controversial because investigators do not all agree that antioxidants confer protection [62, 63], nor do all agree on the presence of increased lipid peroxidation or ROS generation in ischemia [62, 63]. Some researchers have reported that exogenously administered superoxide dismutase protects the kidney [44]. Allopurinol, which inhibits xanthine oxidase, also has been found protective by some investigators [64] and ineffectual by others [63, 65]. Glutathione, normally present in high amounts in tubular cells, can react with and neutralize ROS. Cellular glutathione levels fall with ischemia [66], and reduced cellular glutathione levels sensitize cells to oxidative stress [67]. Protective effects of glutathione have been reported, although it remains controversial as to whether these effects are due to the antioxidant characteristics of this compound or due to the generation of glycine, its metabolic product, independent of ROS scavenging. As with other ROS scavengers, glutathione administration has yielded inconsistent results [68, 69].

Acidosis. Ischemia stimulates glycolysis, increasing the generation of protons, and leading to a reduction in renal pH [70]. The reduced pH inhibits glycolysis by inhibiting phosphofructokinase, the rate-limiting step in glycolysis. Although severe acidosis can damage the kidney, we have demonstrated in hepatocytes and renal tubular cells that mild intracellular acidosis (extracellular pH 6.9) protects cells against anoxia and substrate deprivation in vitro [71, 72]. Weinberg also found that acidosis protects renal tubular cells [73]. This protection is associated with the prevention of any increase in total cellular Ca^{2+} [72]. The mechanism of protection is not clear but might relate in part to decreased phospholipase activity, given that some renal phospholipase A_2 enzymes have alkaline pH optima [74, 75]. Acidosis stabilizes cell membranes [76]. When blood flow and oxygen delivery are re-established, the extracellular pH rapidly returns to normal. It is possible that the increase in intracellular pH on reperfusion enhances phospholipase A_2 activity. Increased activity of phospholipase A_2 might lead to further alteration in membrane structure and secondarily to increased Ca^{2+} permeability, which in turn would further stimulate phospholipase A_2 activation and perpetuate a positive-feedback process.

Calcium. Damaged tissues are known to accumulate calcium, and dead cells are Ca^{2+}-laden. As increased levels of cellular Ca^{2+} can be implicated in a number of processes that are detrimental to the cell, some investigators have proposed that Ca^{2+} is a principal mediator of cellular injury associated with ischemia [77]. Everyone agrees that damaged cells and tissues accumulate Ca^{2+}, but considerable controversy exists as to whether the increased levels of Ca^{2+} *cause* ischemic cell and tissue injury or are a *consequence* of the injury caused by other factors.

A large Ca^{2+} concentration gradient is maintained between the cytosolic compartment and the extracellular milieu of normal eukaryotic cells. A 10,000-fold concentration gradient (1 mM extracellular to 100 nM intracellular free calcium concentration) is maintained by energy-dependent calcium extrusion mechanisms and low permeability of the plasma membrane to Ca^{2+}. With ATP depletion due to ischemia, Ca^{2+} extrusion from the cell via Ca^{2+} ATPases is inhibited [78]. Furthermore, as ATP levels fall, the activity of the sodium-potassium ATPase pump on the plasma membrane decreases and raises the intracellular sodium concentration. The resultant decrease in the trans-membrane sodium concentration gradient potentiates Ca^{2+} entry into the cell via the sodium-calcium exchanger [79, 80]. Calcium that accumulates in cells can be taken up into non-mitochondrial as well as mitochondrial compartments. Under normal conditions, the non-mitochondrial storage sites play a much more important role in the buffering of calcium than do the mitochondria [81]. If large amounts of Ca^{2+} enter the cells, however, the mitochondria become more important storage sites, representing a very-high-capacity system for buffering Ca^{2+}. Very large amounts of Ca^{2+} can be taken up into mitochondria in the presence of ATP. Small amounts of mitochondrial uptake of Ca^{2+} produce little functional damage to these organelles. Larger amounts of Ca^{2+} uptake, however, cause mitochondrial swelling [82], uncoupling of oxidative phosphorylation [83], and the release of free fatty acids, which themselves uncouple oxidative phosphorylation [84].

Ionized calcium can be detrimental to cells in a number of additional ways. An increase in intracellular Ca^{2+} stimulates plasma and endoplasmic/sarcoplasmic reticulum Ca^{2+}-ATPases. The increased activity of these ATPases increases energy consumption and further contributes to ATP depletion. An increase in cytosolic free Ca^{2+} concentration activates Ca^{2+}-dependent proteases and phospholipases; these enzymes lead to proteolysis and membrane disruption. Elevated Ca^{2+} levels also can disrupt the cell cytoskeleton [85].

If Ca^{2+} participates in the cell injury that characterizes ischemia, it is reasonable (albeit not essential) to predict that cellular Ca^{2+} rises prior to the initiation of irreversible cell damage. Using isolated cardiac myocytes exposed to 30 minutes of anoxia and substrate deprivation, we found that cytosolic free Ca^{2+} and total cellular and mitochondrial Ca^{2+} content did not increase despite evidence for irreversible contracture and mitochondrial dysfunction [81, 86, 87]. Other investigators have measured increases in intracellular Ca^{2+} in anoxic renal tubules or renal cells in culture exposed to metabolic inhibitors (chemical anoxia). Snowdowne and colleagues found small (less then threefold) increases in cytosolic free Ca^{2+} in anoxic isolated cardiac cells [88] and cultured kidney

cells [89]. When they reduced extracellular sodium concentration, the same investigators found much larger increases in cytosolic free Ca^{2+} concentration (12-fold), which were not associated with an alteration in cell viability. Mandel and colleagues evaluated renal tubular mitochondrial Ca^{2+} loading in anoxia and hypoxia [90] and found that Ca^{2+} accumulates in the mitochondria during severe hypoxia but not during anoxia. The Ca^{2+} accumulation with hypoxia, however, is readily reversible on reoxygenation and appears to be unrelated to the respiratory dysfunction elicited by short-term hypoxia or anoxia. Cytosolic free Ca^{2+} was unchanged after 40 minutes of anoxia, at a time when respiration was impaired and the plasma membrane was damaged [91]. Using electron microprobe analysis techniques, they found no anoxia-induced changes in Ca^{2+} in any cellular compartments. Consistent with the findings in cardiac myocytes and renal tubules, Lemasters et al found a loss of cellular membrane integrity as well as mitochondrial dysfunction in the absence of a significant change in cytosolic free Ca^{2+} concentration in hepatocytes [92]. Thus, even those investigators who have found an increase in cytosolic free Ca^{2+} with anoxia have reported changes in Ca^{2+} concentration that are much smaller than those acute changes observed with physiologic processes such as cell fertilization or contraction (10-fold to 25-fold) [93, 94], hormonal stimulation (8-fold) [95], or extracellular sodium depletion (12-fold) [80].

Others have argued for a primary role of Ca^{2+} in renal tubular cell injury based on data obtained in experiments in which Ca^{2+} was removed from the bathing medium or cells were exposed to calcium-channel blockers. If Ca^{2+} is a primary mediator of tissue damage associated with ischemia, then maneuvers designed to prevent Ca^{2+} entry into the cell would be expected to prevent anoxic and ischemic cell injury. Wilson and Schrier, using vital dye exclusion as a measure of viability, found that the viability of proximal tubular cells in primary culture that were exposed to anoxia was greater at 48 hours if Ca^{2+} had been removed from the media for the initial 2 hours post anoxia [96]. Takano and coworkers found that exposure of isolated renal tubules to 30 minutes of anoxia in the presence of a low-Ca^{2+} medium (2 μM) reduced LDH release during the anoxic period but increased LDH release during reoxygenation [97]. By contrast, Smith and colleagues found that hepatocytes were more susceptible to toxic injury in the absence of Ca^{2+} than in its presence [98]. In our own studies with isolated adult rat ventricular cells exposed to anoxia, we found greater changes in electrolyte composition and decreased ability to incorporate ^{14}C-phenylalanine into cell protein if the anoxic incubation was carried out in a bath with Ca^{2+} less than 10 μM [86].

Calcium-channel blockers have been used extensively to explore the role of Ca^{2+} in ischemic cell injury. Many studies in a variety of organ systems have demonstrated a protective effect of this pharmacologic class of agents [99–101]. These experiments are of obvious interest because of the widespread availability and clinical use of these agents. In fact, one can demonstrate that the protection afforded by these agents is accompanied by less calcium loading of mitochondria, preservation of mitochondrial function [102], and less overall accumulation of calcium intracellularly.

Controversy has arisen as to the mechanisms of protection of calcium-channel-blocking agents. Some investigators have interpreted protective effects as due to prevention of calcium overload of cells with a resultant preservation of viability [100]. In a model of fixed ischemia in vivo and in the isolated rat kidney, under conditions in which verapamil could not alter renal blood flow, we found that this agent did not protect the kidney [33]. Verapamil did protect against ischemic injury in a norepinephrine model of acute renal failure [33] and we concluded that the protection afforded by verapamil was due to an inhibition of norepinephrine-induced renal vasoconstriction. Consistent with the conclusion that verapamil does not directly protect the renal epithelial cell are the observations by Weinberg and colleagues that marginal effects are seen in the isolated tubule preparation, even at very high doses of verapamil [103].

We found that neither verapamil nor nifedipine protected isolated non-contracting heart cells [104] or renal tubules (unpublished data) against injury associated with 30 to 40 minutes of anoxia. On the other hand, when cardiac myocytes were paced to rhythmically contract in an anoxic environment, verapamil and nifedipine conferred protection by decreasing the cellular contractile activity [104]. Calcium-channel blockers—in particular, diltiazem and verapamil—have been used in an attempt to decrease the incidence of acute renal failure in the renal allograft. These agents have been administered to both donor and recipient, and the incidence of acute renal failure accounting for delayed graft function has decreased [101]. The drugs were given to patients who also were receiving cyclosporine, and it is not clear whether the effect on graft function was related to an independent effect of the calcium-channel blocker, that is, its ability to block cyclosporine toxicity [105, 106]. Vasoconstriction likely plays an important role in both allograft acute tubular necrosis and cyclosporine toxicity, so one might expect that an agent that vasodilates the renal vascular beds might protect against acute renal failure.

An additional complicating feature in the use of calcium-channel-blocking agents to test the role of Ca^{2+} in ischemic injury is that these agents have effects other than their well-known effects on the slow Ca^{2+} channel. These compounds antagonize calmodulin and phosphodiesterases [107, 108], and they probably also have nonspecific "local anesthetic" or "membrane-stabilizing" actions brought about by their interaction with hydrophobic regions of the membrane bilayer [109].

In summary, it remains unclear how important an increase in cytosolic free Ca^{2+} is to the irreversible renal cell injury that results from ischemia and reperfusion. Clearly, increases in smooth muscle Ca^{2+} concentration lead to vasoconstriction, which can compromise local blood flow. In my opinion, however, increases in cytosolic Ca^{2+} concentrations are neither necessary nor sufficient to explain irreversible tubular cell injury. This is not to say that Ca^{2+} is unimportant. Under certain circumstances, increases in cytosolic Ca^{2+} concentration potentiate the cellular insult. For example, it is possible that mitochondrial loading with amounts of Ca^{2+} that are not of themselves detrimental will potentiate the toxicity associated with ROS, as we have reported [48]. One also should consider that enzymes that participate in the cellular injury associated with ischemia can be modified so as to be more sensitive to Ca^{2+}. Under these circumstances, Ca^{2+} might play an important role without there being any significant increase in its concentration. For example, the Ca^{2+} sensitivity of phospholipase A_2 is increased under in-vitro conditions that mimic brain

ischemia [110]. If this is the case, a covalent modification of the enzyme may result in "Ca^{2+}-dependent" activation without an increase in cytosolic free calcium concentration. It would follow that perhaps the more effective pharmacologic intervention would be directed toward the inhibition of processes that produce this stable modification of the enzyme rather than directed toward modifying Ca^{2+} handling by the cell.

Phospholipases. Phospholipase A_2s (PLA_2) comprise a family of enzymes that hydrolyze the acyl bond at the sn-2 position of phospholipids to generate free fatty acids and lysophospholipids. I have recently reviewed the various forms of PLA_2 enzymes and the roles that they play in signal transduction and other cellular processes [75]; PLA_2 likely plays an important role in ischemic cellular injury [20, 21]. Phospholipid degradation contributes to ischemic tissue injury in the kidney [111], brain [112], heart [113], intestine [114], and liver [115]. Phospholipase A_2s can contribute to cell injury in many ways: (*1*) The PLA_2-induced changes in phospholipid integrity and the toxic actions of free fatty acids and lysophospholipids alter plasma membrane and mitochondrial membrane permeability properties and bioenergetic capacities. (*2*) The lipid peroxidation that occurs with ischemia and reperfusion causes an increase in susceptibility of cellular membranes to PLA_2 [116]. (*3*) As one of the PLA_2 products, arachidonic acid, is converted to eicosanoids by the PGH synthase and lipoxygenase enzymes, reactive oxygen species are generated. (*4*) The eicosanoid products of arachidonic acid metabolism are vasoactive and chemotactic for neutrophils, which in turn, may contribute to the tissue injury [53]. (*5*) In addition, when the lipid 1-O-alkyl-2-acyl-phosphorylcholine is the substrate for PLA_2, the product becomes the precursor for platelet activating factor, which can activate platelets and cause capillary occlusion.

The extent of the contribution of PLA_2 to cell injury associated with ischemia is controversial [21, 117]. Until recently, much of the evidence supporting a role for PLA_2 was indirect and was based on measured increases in free fatty acids in post-ischemic tissues or on the protection afforded by poorly selective PLA_2 inhibitors. The role of Ca^{2+} in activating PLA_2 was also unclear; many forms of PLA_2 require very high Ca^{2+} concentrations for activation, and the Ca^{2+} concentration sensitivity of intracellular forms of PLA_2 was not well characterized. Venkatachalam et al reported that unesterified fatty acids accumulate to very high levels when ATP levels drop below a critical threshold in renal epithelial cells in culture [118]. The levels to which free fatty acids accumulated correlated positively with the extent of cell injury. A non-specific PLA_2 inhibitor, quinacrine, protects against phospholipid degradation, results in a reduction in infarct size after coronary artery occlusion in rats [113], and also partially preserves the mucosal permeability barrier in ischemic and reperfused intestine [101]. A 4-thiazolidinone compound (LY178002) inhibits multiple enzymes in the arachidonic acid metabolic cascade, including PGH synthase, 5-lipoxygenase, and PLA_2 as well as lipid peroxidation, and protects the rat brain exposed to transient global forebrain ischemia [119].

The mitochondria are particularly important sites of PLA_2 action, and PLA_2 activation might have important implications for cellular energy metabolism [48, 120]. Changes in mitochondrial membrane integrity would be expected to impair ATP generation and hence reduce the energy currency available for recovery processes [121]. We found that PLA_2 activation was responsible for electron transport chain damage localized to NADH coenzyme Q reductase, decreased F_1-ATPase function, decreased ADP translocase activity, and increased mitochondrial membrane permeability to hydrogen ions, when mitochondria were exposed to Ca^{2+} and reactive oxygen species [48]. Mitochondria, enriched with polyunsaturated fatty acids, were more susceptible to injury from exposure to Ca^{2+} and ROS; this susceptibility correlated positively with enhanced PLA_2 activity [122].

Data from other laboratories also support the view that PLA_2 plays an important role in mitochondrial injury. The addition of exogenous PLA_2 to hypoxic proximal tubules results in severe cellular injury and significant decreases in uncoupled respiratory rates of the tubules [123]. An understanding of the role of PLA_2 in ischemic injury requires that we (*1*) characterize PLA_2 activity in the organ of interest with regard to apparent molecular size and substrate specificity; (*2*) determine whether PLA_2 enzymatic activity itself is modulated by ischemia and reperfusion; and (*3*) evaluate how potential modulating factors, including calcium, pH, protein kinase C, and ROS affect PLA_2 activity. I will address each of these issues by focusing on our own experiments using rat kidney, gerbil brain, isolated rat kidney mitochondria, and isolated rat cortical neurons in culture.

Recently we characterized PLA_2 activity in cytosolic, mitochondrial, and microsomal fractions of rat kidneys [74]. At least two forms of PLA_2 activity were present in the cytosolic fraction. A high-molecular-weight form, active against phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was purified and found to have a molecular mass of approximately 110 kD [124]. A smaller form with a molecular mass of approximately 14 kD that is active against PE also was found in the cytosolic fraction. In the mitochondrial and microsomal fractions, a single dominant form (Mr $\approx$ 14 kD) was active against both PC and PE. As in the kidney, two forms of PLA_2 were present in the cytosolic fraction of gerbil brain: a high-molecular-weight form, active against PC and PE, and a smaller form with a molecular mass of approximately 14 kD, active against PE. In the mitochondrial and microsomal fractions, again a single dominant form (Mr $\approx$ 14 kD) was active against both PC and PE [125].

We measured the PLA_2 activity of cytosolic, mitochondrial, and microsomal extracts of rat kidney [74] and gerbil brain [125] after ischemia and reperfusion and compared these results with those from control tissue extracts. Each of the extracts was assayed under identical free Ca^{2+} concentrations and at pH 7.5. In kidneys exposed to 45 minutes of clamp-induced ischemia with one hour of reperfusion, we noted increased cytosolic, mitochondrial, and microsomal activity. We determined PLA_2 enzymatic activity in gerbil brain after 10 minutes of common carotid occlusion followed by 10 minutes of reperfusion. The PLA_2 specific and total activities increased significantly in each extract when PC or PE was used as a substrate. In the brain, the cytosolic PLA_2 activity after ischemia and reperfusion increased when PC, PE, phosphatidylinositol (PI), or 1-hexadecyl-2-arachidonyl-PC was used as a substrate. The highest PLA_2 specific activity was found in the presence of 1-hexadecyl-2-arachidonyl-PC. Ischemia and reperfusion did not change

the gel filtration elution patterns of PLA_2 activity of the various forms of the enzyme in either kidney or brain.

In both rat kidney and gerbil brain, cytosolic PLA_2 activity increased when we raised the Ca^{2+} concentration from 100 nM (baseline cytosolic Ca^{2+} concentration in renal epithelial cells and neurons) to 200–300 nM, values well within the range reached in response to physiologic and pathophysiologic stimuli [95, 126]. Mitochondrial PLA_2 activity also was Ca^{2+} concentration-dependent, but activity was not increased until $[Ca^{2+}]$ was increased to levels greater than 1 μM. Activity increased, however, as $[Ca^{2+}]$ increased from 1 to 5 μM, values within the range of variation of mitochondrial matrix $[Ca^{2+}]$ under physiologic and pathophysiologic states [90, 127].

In both kidney and brain, cytosolic, mitochondrial, and microsomal PLA_2 activities were optimal at pH 8.5. Ischemia and reperfusion did not alter the pH optima of cytosolic or mitochondrial PLA_2 activity. This alkaline pH optimum may explain the acidosis-associated protection against anoxic injury that we reported [61] and have discussed previously.

Thus, rat kidney and gerbil brain have various forms of Ca^{2+}-dependent PLA_2 activity. Ischemia and reperfusion result in stable activation of both soluble and membrane-associated forms. This stable activation of PLA_2 may play an important role in cellular injury associated with ischemia and reperfusion and may account for continued phospholipid degradation after the acute insult is reversed.

Additional forms of PLA_2 might be present in the kidney. In a preliminary report, Morrison and Irwin noted the presence of a Ca^{2+}-dependent, PE-specific, cytosolic PLA_2 in the rabbit renal cortex [128]. Portila and colleagues reported a 40 kD form that is immunologically related to the pancreatic group I PLA_2; its activity is increased when rabbit renal proximal tubules are exposed to anoxia [129].

In the heart, ischemia has a significant effect on a Ca^{2+}-independent form of PLA_2, which is selective for plasmalogens [130]. The kidney also contains large amounts of plasmalogens. When ^{14}C-ethanolamine is infused into rats, there is greater incorporation of radioactivity into the plasmalogen fraction than into phosphatidylethanolamine. This preferential incorporation is greater in the kidney than in the heart or liver [131]. As yet the literature contains no report of a renal PLA_2 that has a preference for plasmalogens as substrates.

While elevated cytosolic Ca^{2+} concentration might be important for activating the PLA_2 enzymes in vivo with ischemia, the preservation of increased activity in vitro under conditions of our assay, in which Ca^{2+} is fixed, suggests a stable modification of the enzymes. Thus ischemia and reperfusion may produce a covalent modification of the enzyme such that PLA_2 activation prevails even if $[Ca^{2+}]$ levels return to baseline values in vivo after ischemia. This persistent PLA_2 activation may contribute to the accumulation of arachidonic acid and metabolic products of arachidonic acid in tissues after reperfusion [132].

Protein kinase C (PKC) may regulate PLA_2 via phosphorylation [126]. Evidence suggests that PKC is activated during ischemia [133]; phosphorylation of PLA_2 might enhance its activity. In the mesangial cell, PKC activation with phorbol esters increases the Ca^{2+} sensitivity of PLA_2 activity [126]. Recent studies suggest that a mitogen-activated protein kinase, MAP kinase, is able to directly activate the 110 kD PLA_2 [134].

Using glutamate stimulation of isolated rat brain cortical neurons, we further found evidence for increased Ca^{2+} sensitivity of PLA_2 in the cell culture model of ischemia. Excitatory amino acid toxicity probably is of paramount importance in the neuronal death that occurs with brain ischemia. Glutamate stimulation of isolated neurons raises cytosolic Ca^{2+} concentration [135], PKC activity, and PLA_2 activity in cytosolic extracts of the cells [110]. The characteristics of this PLA_2 activity approximate those of rat kidney and gerbil brain cytosolic extracts. In the post-glutamate extracts, the Ca^{2+} sensitivity of the small-molecular-weight form of PLA_2 is increased [110].

In addition to the possible mediation of Ca^{2+} and PKC-related ischemic cellular injury, PLA_2 is likely an important mediator of the injury that occurs in response to ROS in the presence of increased amounts of Ca^{2+}. As I previously stated, in isolated rat kidney mitochondria, the damage to the electron transport chain, F_1-ATPase, and adenine nucleotide translocase, observed when mitochondria are exposed to Ca^{2+} and reactive oxygen species, was substantially reduced by PLA_2 inhibitors [48]. The alkaline pH optimum of the mitochondrial form of the enzyme suggests that PLA_2 activity is increased in the alkaline environment of the mitochondrial matrix.

In summary, multiple forms of PLA_2 are present in the cytosolic and membrane (mitochondrial and microsomal) compartments of kidney and brain cells. The Ca^{2+} concentration sensitivities of cytosolic and membrane-bound PLA_2 activities indicate that the enzymes likely are regulated by Ca^{2+} in vivo. After ischemia and reperfusion in the kidney and brain, cytosolic, mitochondrial, and microsomal PLA_2 enzymatic activities were increased. Although changes in $[Ca^{2+}]$ might play a permissive role in these stable modifications of enzymatic activity, the changes in PLA_2 activity probably are not explained by changes in $[Ca^{2+}]$ alone and suggest that other regulatory influences contribute significantly to PLA_2 activation and mediation of cellular injury after an ischemic insult.

Amino acids. Using renal tubules and renal epithelial cells in culture, Weinberg and colleagues found that certain amino acids can protect against injury associated with increases in cytosolic free $[Ca^{2+}]$, ROS, ATP depletion, and sodium-potassium ATPase inhibition [136–138]; low millimolar concentrations are necessary and glycine is most effective. The mechanism of this protection is not known. Depletion of amino acids such as glycine, which permeate cell membranes, might be relevant clinically, especially in the transplanted kidney, which can become depleted of glycine during storage [139].

Proteases. Renal brush-border membranes are rich in proteases [140]. The involvement of these proteases, which include meprin, endopeptidase 24.11, and exopeptidases, and the ionized Ca^{2+}-dependent neutral cysteine proteinases, calpains [141], in ischemic injury is not well understood.

Synergy. A complicating feature that frustrates biologists trying to understand any biologic system is its inherent complexity. Such is also the case with the study of cell injury and cell death. It is clear that many of the factors we have discussed do not work in isolation. They interact in complicated, poorly understood ways. These interactions, together with the nonspecificity of interventions introduced to inhibit a particular injurious influence, and the difficulty in defining the "point of no return," all conspire to make it difficult to define the most

important factors determining cell injury and death. The pessimist concludes from this complexity that many factors are important and the prevention of cell death, which will depend on interference with multiple mechanisms, will not be feasible at a clinical level. The optimist, like me, argues that one or two of these factors are most important and that even when synergy occurs, one or two factors will predominate. Hence therapeutic intervention directed at these one or two factors will be successful in preventing cell death.

One example of synergy that I already mentioned is the detrimental effects on mitochondrial function that arise due to interactions of Ca^{2+} and phospholipase A_2 and ROS [48]. Increased mitochondrial Ca^{2+} and ROS can act synergistically to confer a degree of toxicity that is far greater than that expected simply from the additive toxicities of the two agents. For example, we evaluated in vitro the damage produced to rat renal cortical mitochondria after exposure to Ca^{2+} alone, ROS generated by hypoxanthine, xanthine oxidase and iron, or the combination of Ca^{2+} and ROS [48]. Mitochondrial electron transport chain integrity, ATP synthetase (F_1F_0ATPase), and adenine nucleotide translocase were evaluated. Mitochondria exposed to ROS alone sustained an increase in substrate-supported (state 4) respiration due to increased mitochondrial membrane permeability, decreased ADP-stimulated (state 3) respiration, and a 50% reduction in the respiratory control ratio (state 3/state 4); F_1F_0ATPase was inhibited, but neither the electron transport chain nor the ATP-ADP translocase was damaged. In contrast, after exposure of mitochondria to small amounts of Ca^{2+} (30 nmol/mg mitochondrial protein), which by itself did not change mitochondrial function, the addition of ROS caused a greater increase in mitochondrial membrane permeability, a major functional defect in the electron transport chain, a marked decrease in F_1F_0ATPase and adenine nucleotide translocase activity, and a complete uncoupling of oxidative phosphorylation. The mechanism of this Ca^{2+} potentiation of ROS damage is, at least in part, via activation of phospholipase A_2. Dibucaine, a phospholipase A_2 inhibitor, partially protected the electron transport chain, F_1F_0ATPase, and adenine nucleotide translocase from damage.

Apoptosis: Does it play a role in ischemic cell injury?

Until recently, the study of cell death received much less attention than the study of cell proliferation and differentiation. In general, cell death has been considered less interesting for study because it has been seen as a degenerative process characterized by necrosis. It is now recognized, however, that certain types of cell death are finely controlled by active processes. For example, during metamorphosis and development, "programmed cell death" represents an important cellular response to physiologic stimuli that allow for the proper formation of the organism. "Cell death" genes have been identified in the nematode *Caenorhabditis elegans* [142]. Testosterone withdrawal results in involution of the prostate by a process involving programmed cell death [143]. Abnormalities in the control mechanisms for programmed cell death might explain certain neurodegenerative diseases [144].

Most of the attention devoted to ischemic cell death has focused on necrosis. The pathologic features of necrosis are very different from those of programmed cell death. The latter has been called "apoptosis," a term coined by Kerr et al, who attribute the derivation of the term to Professor James Cormack of the University of Aberdeen [145]. "Apoptosis" is a Greek term that means "dropping off" or "falling off," as say, petals from a flower or leaves from a tree. Pathologically, apoptosis is characterized by chromatin condensation at the periphery of the nucleus, condensation of the cytosol, diappearance of microvilli, breakdown of epithelial desmosomal attachments, and cell surface protuberance, followed by disruption of the nucleus, and finally the blebbing off of cell surface protuberances around condensed cytosol and perhaps some nuclear fragments, thus generating spherical or ovoid "apoptotic bodies" [146]. Macrophages or epithelial cells can phagocytose these short-lived apoptotic bodies, which can be seen by light microscopy in tissue for only 12 to 18 hours. The biochemical correlate of chromatin condensation is cleavage of double-stranded DNA at the linker regions between nucleosomes, resulting in fragments of approximately 185 bp [147]. Agarose gel electrophoresis reveals a characteristic "ladder pattern." By contrast, necrosis is characterized by random DNA breakdown with a diffuse pattern seen on gel electrophoresis. The endonuclease responsible for this electrophoretic pattern typical of apoptosis has not been well characterized. A candidate enzyme is an endonuclease that is activated by calcium in thymocytes [148]. In fact, under certain conditions, elevations in intracellular calcium concentrations can induce apoptosis [149]. It is therefore possible that Ca^{2+} is involved in the activation of apoptosis under pathophysiologic conditions. Under many conditions of cell injury, one cell might undergo cell death while an adjacent cell proliferates to replace the damaged cell. It is interesting that phorbol esters can change the effects of Ca^{2+} elevation from cell death to proliferation in thymocytes [149]. Clearly, the apoptotic process is under fine control.

I propose that apoptosis plays an important role in ischemic cell injury. An understanding of the role of apoptosis in ischemic cell injury likely would facilitate pharmacologic intervention directed toward the active cellular processes that occur during the post-ischemic period and during periods of chronic ischemia such as during renal artery stenosis. The question then becomes: Is there any evidence for apoptosis with ischemic acute renal failure? Gobé et al studied renal atrophy that accompanies experimental renal artery stenosis [150]. During the first 2 to 8 days after the partially occluding clip was placed around the renal artery, they found pathologic evidence for both necrosis and apoptosis of renal epithelial cells. From 10 to 28 days after clip placement, when renal mass was markedly reduced, cell death continued, but only apoptosis was observed. After unilateral renal atrophy had occurred, removal of the contralateral kidney resulted in marked enlargement of the atrophic kidney; thus the atrophic process was reversible. An understanding of the factors involved in the reversal of the apoptotic process might allow us to prevent the ischemic tissue atrophy in the first place. Schumer and colleagues examined renal tissue at various times after 5, 30, or 45 minutes of ischemia [151]. A small number of apoptotic bodies were found 24 and 48 hours after reperfusion following 5 minutes of ischemia; there was no associated necrosis. In the kidneys clamped for 30 or 45 minutes, apoptosis was found at 12, 24, and 48 hours of reperfusion. A ladder pattern of DNA fragmentation also was observed 24 hours after 30 minutes of ischemia. With increasing periods of ischemia, evidence for necrosis

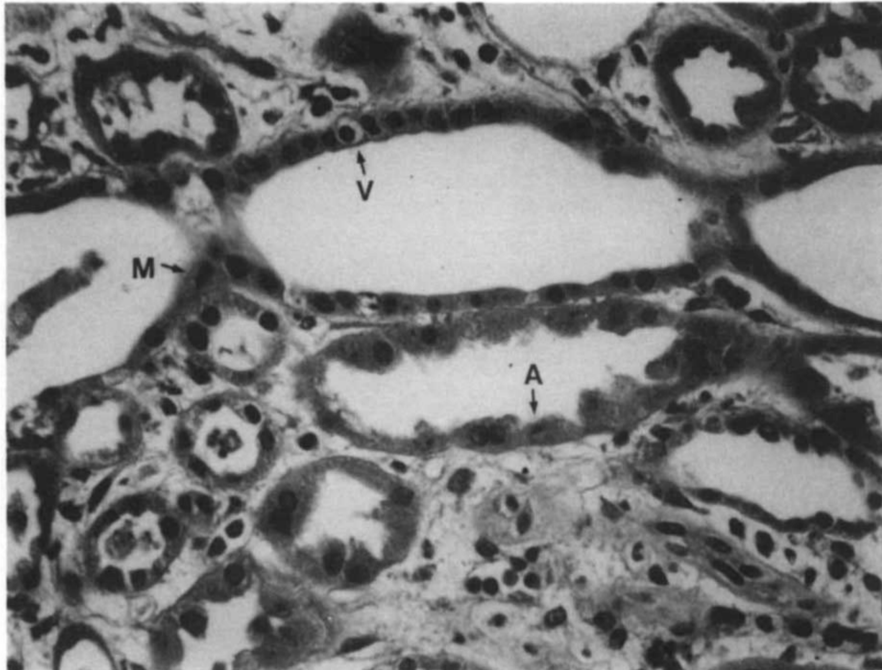


Fig. 2. *Apoptosis in patient with acute renal failure.* A represents an apoptotic nucleus, V represents a vacuole, and M represents a mitotic figure.

increased. Figure 2 demonstrates apoptosis in a renal biopsy specimen of a patient with acute renal failure.

Ischemia and reperfusion are accompanied by the induction of various genes, including the gene encoding sulfated glycoprotein-2 (SGP-2) [152], also called clusterin and TRPM-2, a gene that has been implicated in apoptosis in the prostate [143]. We have found that other genes, *Egr*-1 and c-*fos*, whose expression is induced under many situations in which cells are stimulated to proliferate, are also induced with ischemia and reperfusion, and that protein levels of *Egr*-1 increase to very high levels in thick ascending limb nuclei [153, 154] (Fig. 3). The control mechanisms responsible for the decision of a cell to die via an apoptotic process, or to proliferate to replace adjacent cells that have died will, I hope, become better understood as cellular signaling and genetic mechanisms for "programmed cell death" are better defined.

Apoptosis has been documented to be particularly prevalent in post-transplant acute tubular necrosis [155]. Whether this finding reflects fundamental differences in the pathophysiology of ischemic acute renal failure between a native kidney and a transplanted organ is not clear [155]. Joel Weinberg and I reviewed the pathophysiologic factors implicated in ischemic acute renal failure in the transplanted kidney [32].

Summary

The pathophysiology of ischemic acute renal failure is complex. The morbidity and mortality associated with the clinical problem remain unacceptably high; therefore it is imperative that we continue a diligent search for a better understanding of the mechanisms responsible for ischemic cell death. Such knowledge might lead to therapeutic interventions directed at the most important pathophysiologic factors responsible for the tubular cell's demise and could have applications to other organs frequently ravaged by the consequences of ischemia—the heart and brain. At the same time, we must continue to explore basic mechanisms responsible for renal repair, as the kidney has the potential to completely recover, a property not shared by heart or brain. By understanding the injury and repair mechanisms [19], I hope we will be able to prevent the tissue damage and, when this is unsuccessful, hasten the repair process or initiate it in kidneys where it otherwise would not occur.

Questions and answers

Dr. Nicolaos E. Madias (*Chief, Division of Nephrology, New England Medical Center, Boston, Massachusetts*): I was interested in your finding that acidosis might protect renal tubular cells from anoxic injury. You proposed as a possible explanation an inhibitory effect on phospholipase A_2 activity. Dr. Hilden, who is in the audience, has shown in preliminary studies induction of heat-shock proteins in the kidney by ischemia and acidosis. I wonder whether such induction could be another mechanism for the protective effect of acidosis. May I ask that you and Dr. Hilden comment on this issue?

Dr. Bonventre: The heat-shock response is remarkably conserved during evolution. Cells exposed to various noxious and potentially lethal stimuli, such as heavy metals, viruses, calcium ionophores, and heat, respond by increasing the synthesis of a group of proteins (the heat-shock or stress proteins), while decreasing the synthesis of most other proteins [156, 157]. We reported that the mRNA levels of a member of the HSP 70 gene family are increased soon after an ischemic insult to the kidney [158]. The mRNA levels are maximal at 3 hours after the renal artery has been clamped for 40 minutes. These data are shown in Figure 4, which presents a Northern blot analysis demonstrating mRNA levels of HSP 70 and comparing them to mRNA levels of *Egr*-1 (also known as Zif-268, Krox-24,

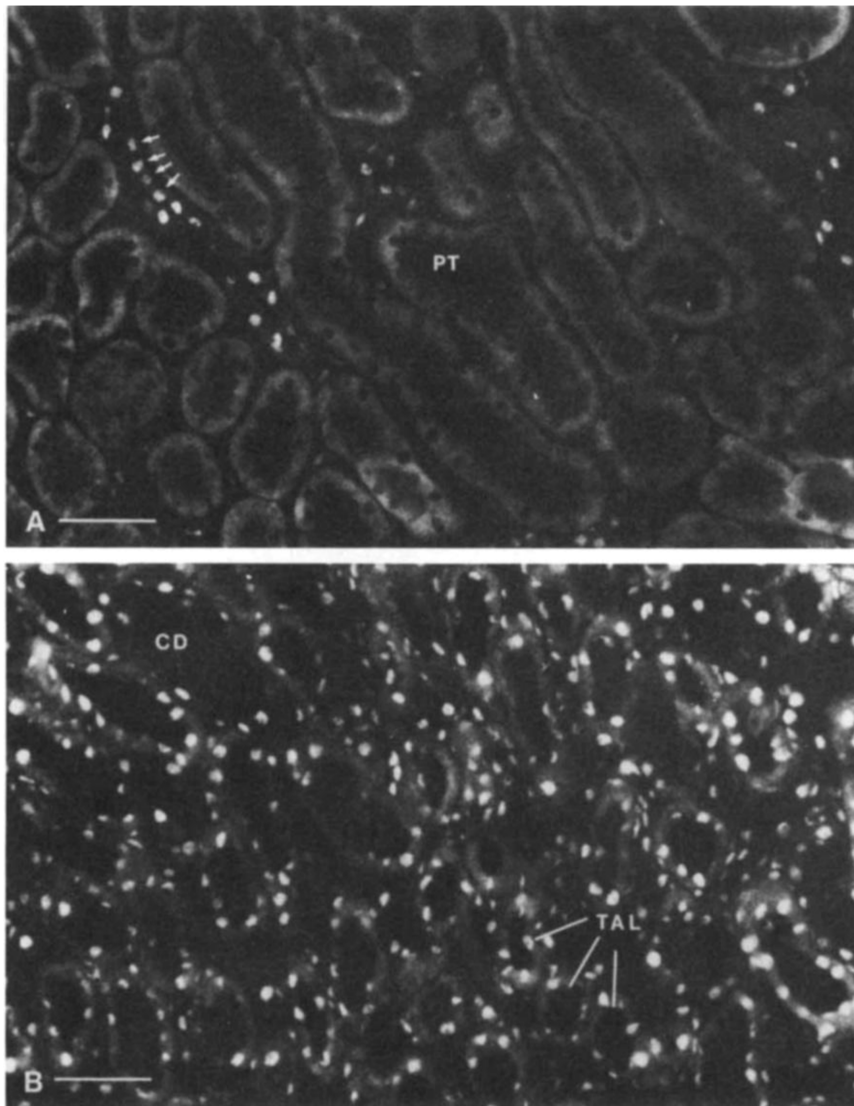


Fig. 3. *Cryostat sections of outer medulla of ischemic rat kidney after one hour of reperfusion, stained with an antibody, to the Egr-1 protein.* (A) Outer strip of outer medulla showing positive staining of nuclei of thick ascending limbs (arrows) and collecting ducts. The nuclei of straight proximal tubules (PT) in the outer stripe are not stained. (B) Inner stripe of outer medulla showing heavy nuclear staining of thick ascending limbs (TAL) and collecting ducts (CD). In addition, the nuclei of some thin limbs and capillaries appear to be fluorescent. Bar = 50 μm (from reference [154]).

NGFI-A, and TIS-8) [159], an early growth response gene that is also markedly and transiently induced with ischemia.

We were most successful in demonstrating a protective effect of this heat-shock response using a model for ischemic injury in the brain [160], in which amino acid excitotoxicity is believed to play a critical pathophysiologic role. We established cultures of cortical neurons and exposed these neurons to glutamate. The glutamate-induced excitotoxicity was inhibited by preheating cells to 42.2°C for 20 minutes. This protection was afforded to cells subsequently exposed to glutamate 3 or 24 hours after they were heated. Protection required new protein synthesis. I would be interested in knowing whether Dr. Hilden has any further comments.

DR. SHIRLEY A. HILDEN (*Division of Nephrology, New England Medical Center*): Dr. Madias, Conrado Johns, and I found that renal function is protected in rats that were treated with heat-shock 2 days before the ischemic insult, at least as demonstrated by a normal creatinine. I'm interested in correlating some of the changes that occur in ischemic kidneys and the synthesis and aggregation of heat-shock proteins, especially in terms of your discussion about the adenine nucleotide changes in ischemia. My results could be explained by an aggregation of heat-shock proteins with proteins that are in the process of being synthesized and require ATP for proper processing. When ATP is reduced by ischemia, the ensuing aggregation of heat-shock proteins might reflect a disturbance of normal protein synthesis and processing.

DR. MADIAS: Wasn't acidosis by itself also an inducer of the heat-shock response?

DR. HILDEN: I'm interested in characterizing different heat-shock proteins in different experimental situations, not just ischemia. The situations we're looking at include acidosis. The protein heat-shock 60 (HSP 60), for example, increased as a function of acidosis. We also saw that acidotic animals that were subsequently treated with ischemia lost this acidosis-induced HSP 60 induction. I'm not sure how that result relates to your observations about acidosis being protective.

DR. BONVENTRE: My working hypothesis is that the acidosis-induced protection against anoxic injury that we observed in kidney and liver is related to an inhibition of phopholipase A_2 activity due to the pH dependency of the cytosolic, mitochondrial, and microsomal PLA_2s in the cell. Dr. Hilden's results

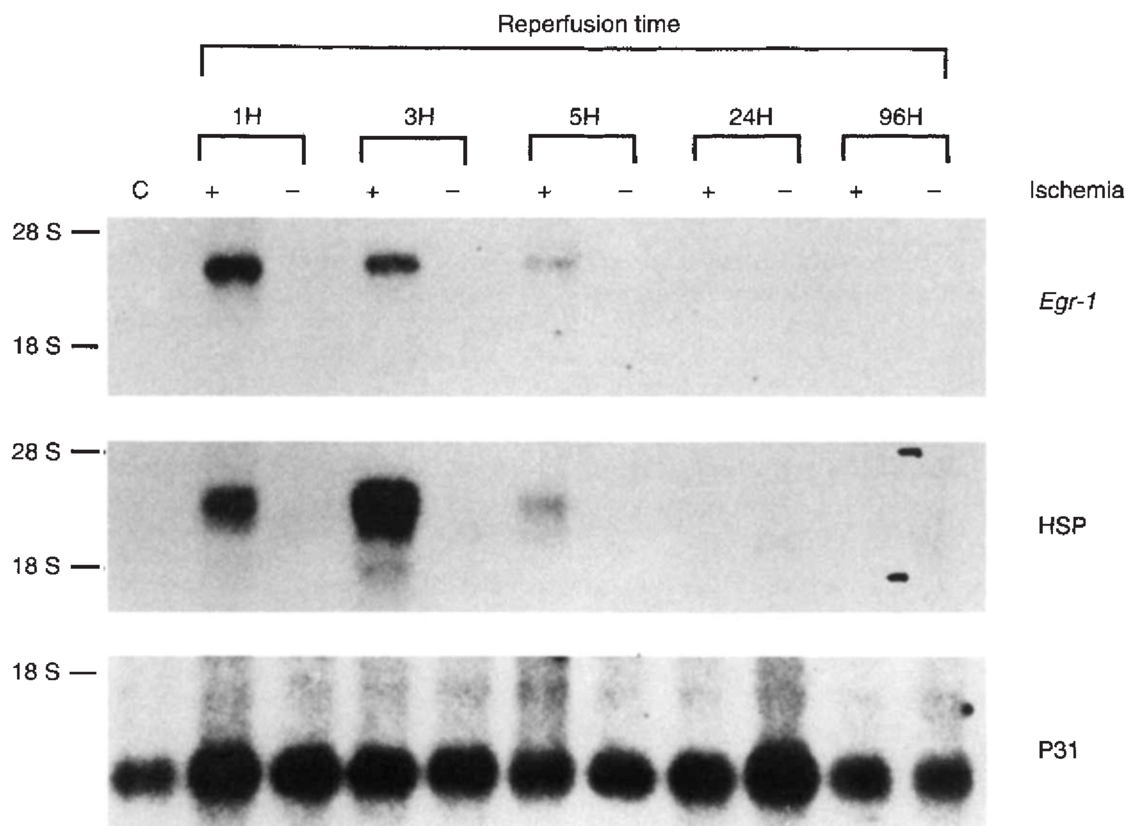


Figure 4. *Northern blot analysis of Egr-1 and HSP-70 mRNA accumulation in rat kidney after 40 min of unilateral ischemia and varying periods of reperfusion.* Total RNA was prepared from both postischemic kidney (+) and contralateral nonischemic (−) kidneys removed at varying times after reperfusion. (C)—RNA obtained from a control kidney obtained from an animal not subjected to ischemia. Blots were hybridized with *Egr*-1 or HSP-70 cDNA probes. The blots also were hybridized with a cDNA probe encoding a "housekeeping" protein (P31). Positions of 18S and 28S mRNA are marked. (From Refs. 154 and 158.)

indicating that acidosis may induce expression of one or more members of the HSP 60 family are possibly relevant to the acidosis-induced protection. The effect of acidosis on expression of DNA binding of a pre-existing heat-shock factor to the HSP 70 heat-shock element was examined by Benjamin et al [161]. Whole-cell extracts were examined in gel shift reactions using an oligonucleotide containing the heat-shock element consensus region. Energy depletion associated with ATP depletion stimulated heat-shock-factor binding, but acidosis alone, without ATP depletion, did not have any effect. I would like to add something that I neglected to mention previously. Together with Drs. Barbara Polla and Yves Donati, who are at the Hôpital Cantonal Universitaire in Geneva, Switzerland, we evaluated whether the heat-shock response protected against acute ischemic injury in rats (unpublished data). We induced the response by raising the temperature of anesthetized rats to 42°C for 20 minutes. This change resulted in a large increase in HSP-70 mRNA levels. Four to six hours later we produced bilateral renal ischemia for 40 minutes and followed renal function by monitoring BUN and creatinine for 7 days post ischemia. We found no protection associated with prior generation of the heat–shock response. Perhaps we would have found protection had we waited a longer period of time between the generation of the heat-shock response and the ischemia. Emami et al reported that accumulation of HSP–72 protein in kidney is maximal within 4 to 6 hours after heat stress and persists for 10 days [162].

Dr. Madias: Other investigators have shown enhanced post-ischemic cardiac ventricular recovery 24 hours after hyperthermic treatment and induction of the 71 kD heat-shock proteins [163].

Dr. John T. Harrington (*Chief of Medicine, Newton-Wellesley Hospital, Newton, Massachusetts*): What do the so-called heat-shock proteins do before they get shocked by heat? Do they have a role in normal cellular metabolism?

Dr. Bonventre: Some constitutive members of the heat-shock family of proteins function in normal cell physiology. For example, the heat-shock cognate HSC 70 aggregates with denatured proteins. It has been proposed that this heat-shock protein normally binds to the heat-shock factor, which is a protein that potentiates the transcription of genes containing heat-shock elements. Under normal circumstances, the heat-shock factor is inactivated by its interaction with HSC 70. When the cell is shocked, the amount of denatured proteins that compete with heat-shock factor for binding to HSC 70 increases; heat-shock factor is released, DNA binding of the factor increases, and heat-shock protein synthesis increases [164]. Under normal conditions, HSC 70 probably acts as a molecular chaperone, delivering nascent unfolded polypeptides made in the cytosol to the rough endoplasmic reticulum and

mitochondria [165, 166]. Another HSP 70 family member, GRP 78, also called BiP, is localized to the lumen of the endoplasmic reticulum and associates with nascent glycoproteins and secretory proteins; BiP presumably prevents abnormal folding and aggregation. Another abundant cellular heat-shock family is the HSP 90 family. HSP 90 associates with steroid receptors, chaperoning the receptor until steroid hormone is available to bind to it [10, 167].

DR. HARRINGTON: It's well known that medullary blood flow is a relatively small percentage of overall renal blood flow. Why, then, does a reduction in outer medullary blood flow have such a profound effect on overall glomerular filtration rate?

DR. BONVENTRE: Although blood flow to the medulla is much less than that of the cortex, the relative changes in blood flow to this area of the kidney post ischemia are quite large. As I indicated, the blood flow decreased to 16% of normal in the outer medulla 60 minutes after ischemia, whereas cortical blood flow was 60% of pre-ischemic levels, and inner medullary flow was 125% of pre-ischemic levels [7]. This decreased outer medullary blood flow has been attributed to the vascular congestion secondary to the outwardly directed swelling of proximal tubular cells [5]. This decreased blood flow, together with the countercurrent exchange of oxygen along the corticomedullary axis, with graded reduction in tissue oxygen tension with increasing distance from the corticomedullary boundary, is likely to result in severe compromise of oxygen delivery to cells of the outer medulla, which depend on oxidative metabolism for their viability [168]. Since most human nephrons have loops of Henle that traverse the outer medulla, nephron damage to the tubular epithelium in this area is likely to have significant consequences in the form of tubular obstruction and backleak in a large number of nephrons.

DR. ANDREW KING (*Division of Nephrology, New England Medical Center*): Most investigators have observed, both in humans and in animal models, a substantial reduction in both renal blood flow and GFR, with a disproportionate fall in GFR. In addition, the autoregulatory ability of the renal vasculature is impaired. Early in your presentation, you said that glomerular endothelial cells were swollen following ischemia. What role do you think the endothelial cells play? Are they potentially factors that could affect renal vascular tone, tubular damage, and tubular recovery? In particular I'm interested in your thoughts about endothelin, as several investigators have shown an increase in renal endothelin production after acute ischemia.

DR. BONVENTRE: The endothelial cells are involved in a central way in the regulation of vascular tone. They produce and release potent vasodilators, including prostacyclin (PGI_2) and endothelium-derived relaxation factor (EDRF) identified as nitric oxide [169]. They also produce potent vasoconstrictive agents including platelet activating factor, platelet-derived growth factor, and endothelin-1 [170].

Endothelin might play an important role in post-ischemic vasoconstriction. Administration of anti-endothelin antibodies into a branch artery of the main renal artery resulted in decreased renal vasoconstriction post ischemia [171].

Other mediators derived from the endothelium also might contribute to the tubular damage. Endothelial cells can produce interleukins or may express leukocyte adhesion molecules on their surface, which might be important for an inflammatory response and probably increases neutrophil infiltration and exacerbates the injury. Finally, factors produced by the endothelium could be important for tissue repair.

DR. MADIAS: As you and Dr. King noted, several experimental studies have shown impaired autoregulation of renal blood flow following ischemia. Indeed, some observations in humans appear to support the clinical relevance of this loss of autoregulation [172]. Could you comment on the significance of recurrent episodes of hypotension, including those related to dialysis, in recurrent ischemic injury to the kidney?

DR. BONVENTRE: The post-ischemic kidney has abnormal autoregulation of renal blood flow. This alteration has been demonstrated in dogs [173] and rats [174]. Thus a subsequent reduction in arterial pressure might be expected to produce a greater decrease in renal blood flow in the post-ischemic kidney than it would in normal kidneys. As you suggest, therefore, the transient hypotension that frequently accompanies hemodialysis in the patient with acute renal failure might result in additional tissue ischemia, further delaying the recovery of the patient. This remains controversial, however, because some investigators have found in experimental animals that a prior episode of ischemia partially protects the kidney against a second ischemic episode [175]. This protection might be due to less susceptibility of regenerating tubular epithelium to ischemia. If recovery from acute renal failure recapitulates aspects of early renal development, the regenerating epithelial cell might be similar to the tubules of young animals, which are known to be more resistant to ischemic injury [176]. Another possible reason for decreased cellular susceptibility to a second ischemic insult is the presence of protective factors in the cell that are induced as a result of the first ischemic episode. Heat-shock proteins are candidates for this type of protective function, as we previously discussed. Another explanation for protection of the post-ischemic kidney against subsequent ischemia is that the increased sodium and osmotic load to each of the remaining functional nephrons is protective. Additional possible reasons for resistance to a second insult include: inability of the damaged kidney to concentrate a toxic substance, decreased responsiveness of the damaged kidney to vasoconstrictive substances, and increased levels of reactive oxygen species scavengers in renal cells [177, 178].

DR. AJAY K. SINGH (*Division of Nephrology, New England Medical Center*): In your excellent presentation, you talked about the role of cell necrosis and about programmed cell death in ischemic injury. You hinted that the apoptosis component of the injury might be accelerated in the ischemic kidney. Is there evidence for this? In particular, are regulatory genes that might be involved in apoptosis up-regulated?

DR. BONVENTRE: One gene that has been implicated in apoptosis is sulfated glycoprotein-2 (SGP-2), also called testosterone-repressed prostate message-2 (TRPM-2) and clusterin. This gene initially was implicated in apoptosis because it is up-regulated when the prostatic cell undergoes "programmed cell death" after testosterone is removed, and in the interdigital region during limb morphogenesis [143]. Expression of this gene, as measured by accumulation of mRNA, is up-regulated in ischemic injury with a time course that is more prolonged than that observed with *Egr*-1 or heat-shock protein genes [179, 180].

DR. SINGH: One could speculate, somewhat simplistically, that cell necrosis results in the release of nuclear factors that

somehow up-regulate *cis*-acting gene-regulating segments important in apoptosis. For this hypothesis to be accurate, one would expect cell necrosis to precede apoptosis. Do you know of any pathologic studies in ischemic renal injury that examine the chronologic relationship between cell necrosis and apoptosis?

DR. BONVENTRE: Drs. Gobé, Axelsen, and Searle, in a pathologic study of chronic renal ischemia, have reported the chronologic relationships between cell necrosis and apoptosis [150]. Ischemia was induced by unilateral renal artery stenosis. During days 2 through 8 after a partially occluding clip was placed on the artery, they found pathologic signs of both necrosis and apoptosis accounting for cell death. From days 10 through 28, by contrast, cell death was due to apoptosis alone. Schummer et al induced ischemia by occlusion of the renal artery for 5, 30, or 45 minutes [151]. A few apoptotic cells were present 12, 24, or 48 hours after a short 5-minute period of ischemia. As the period of ischemia was prolonged to 30 or 45 minutes, the number of apoptotic cells markedly increased at 24 and 48 hours after the ischemia. When DNA was isolated 24 hours after kidneys were clamped for 30 minutes, there was a series of 180–200 base pair fragments, which formed a ladder pattern typical of apoptosis.

DR. BRIAN J. G. PEREIRA (*Division of Nephrology, New England Medical Center*): At the International Symposium on the Diagnosis, Treatment, and Prevention of Acute Renal Failure at Edmonton, the term "acute tubular non-replacement" was introduced to explain the total absence of cells along the tubular epithelium in patients with acute renal failure. What is your opinion about this term?

My second question relates to the patient you described. He clearly had a first insult that led to acute renal failure, recovered, and had a second insult. Could you expand on the relative increase in susceptibility in humans and laboratory animals recovering from acute renal failure?

DR. BONVENTRE: The term "acute tubular non–replacement" seems awkward. It is not clear what triggers replacement of the epithelial cells. A large variation exists in the time from insult to recovery of function and normalization of histology. The period of time that elapses prior to recovery cannot be predicted by the initial character of the insult. I believe that recovery of the epithelium recapitulates developmental paradigms, and a better understanding of the latter will, I hope, lead to a better understanding of recovery from acute renal failure.

I believe that recovery from ischemic acute renal failure is likely regulated by a number of genetic events, some of which involve the up-regulation of genes important for dedifferentiation and proliferation, and others involving the down-regulation of genes that are important for differentiation and growth arrest. Some of the genes involved might be kidney-specific, others not. We recently identified a new transcription factor, which is primarily expressed in the kidney and whose mRNA decreases in content in the kidney after ischemia or following folic acid administration [179]. Folic acid induces acute renal failure with marked tubular cell injury. This cDNA encodes a predicted protein with 13 zinc fingers, which is a motif common to DNA-binding proteins. We have called this new gene *Kid*-1. *Kid*-1 is also developmentally regulated. Its expression increases with increasing time of postnatal development. Therefore its expression is decreased when renal cell proliferation occurs. When a chimeric construct of the non-zinc-finger region of *Kid*-1 and the DNA binding region of GAL4 is transfected into kidney cells along with chloramphenicol acetyl transferase reporter plasmids containing GAL4 binding sites, transcriptional activity is markedly suppressed; this suggests that *Kid*-1 is a transcriptional repressor.

With regard to your second question, I have discussed the controversy surrounding the susceptibility, or lack thereof, of the kidney to a second ischemic insult. It is not straightforward to apply these data in animals to our patient. In this patient the second insult involved sepsis in addition to hypotension. Had the hypotension occurred in the absence of sepsis, the second bout of acute renal failure might not have occurred. There is a strong association between sepsis and acute renal failure, although the reasons for this association are not clearly established. Worldwide, sepsis is an important predisposing factor to the development of acute renal failure both in adult and pediatric populations. Sepsis also has an important influence on outcome in patients with acute renal failure [181]. The pathophysiologic mechanisms responsible for the frequent association of acute renal failure with sepsis are not well understood.

DR. MADIAS: You made several parallelisms between the heart and the kidney and also touched on the role of calcium blockers in ischemic cell injury. Would you address the issue of angiotensin-converting enzyme (ACE) inhibition? Several studies have shown that these agents have a protective effect against the myocardial damage occurring during the reperfusion phase following acute myocardial ischemia [182–184], but I am not aware of such an effect in the kidney.

DR. BONVENTRE: The effects of angiotensin-converting-enzyme inhibition on renal function are quite complex and are very dependent on volume status of the subject and whether renal artery stenosis is present. The latter is always an important consideration since many of our patients with acute ischemic renal failure have vascular disease and may have renal artery disease, which has not necessarily been recognized. Since renin levels are reported to be high in some studies of acute renal failure [185], it is possible that angiotensin II contributes to intrarenal vasoconstriction. It is also known that angiotensin II can reduce the glomerular ultrafiltration coefficient, K_f [186], and this change might contribute to the reduced GFR. Many reports conflict regarding a potential role for the renin-angiotensin system in the pathophysiology of acute ischemic renal failure [187]. These differences may relate to the various models of renal failure used and the inability to distinguish whether protective effects of volume expansion are due to decreased activity of renin-angiotensin system or to independent physiologic effects on other effector mechanisms.

DR. KING: Given the array of cellular events that you outlined in your talk, could you give us any indication whether there is a prescribed pretreatment for patients who are going to undergo an ischemic event? Do you have any recommendations?

DR. BONVENTRE: Since it is clear that volume depletion predisposes to acute ischemic renal failure, any patient who is in danger of undergoing an ischemic insult to the kidney should be well hydrated. In the case of mechanical interruption of blood flow to the kidney, for example, in the repair of an abdominal aortic aneurysm, pre-infusion of mannitol into the kidney may be protective. Pretreatment with mannitol affords

protection in animal models of acute renal failure in which the renal artery is temporarily occluded [32]. Presumably this protection is based on mannitol's ability to prevent cell swelling, thus decreasing outer medullary vascular congestion; in addition, mannitol prevents tubular obstruction because of its diuretic action. Also, mannitol is a hydroxyl radical scavenger. Although mannitol has not been shown to be protective in human acute ischemic renal failure, it is frequently administered prior to renal artery occlusion.

Cell-impermeant solutes have proved effective in the preservation of the kidney ex vivo for transplantation [139]. A number of other agents have been administered to the kidney ex vivo to decrease the incidence of delayed graft function secondary to ischemic injury. Adenosine has been added to the preservation solution to enhance the purine nucleotide precursor pool. Glutathione has been added to counteract the reduction in tissue glutathione levels seen with ischemia [188, 189]. Calcium-channel blockers have been protective in ex-vivo tissue storage [190, 191], likely because they inhibit vasoconstriction, as observed in animal models of norepinephrine-induced acute renal failure [32]. At present, I do not believe that one can recommend with conviction that any of these agents be administered in vivo to assure protection against ischemic injury. The possible exception is mannitol although, as I stated, the data are weak and clinical trials are needed to test its effectiveness. If this agent is administered, one must be careful to avoid volume depletion; acute renal failure has been associated with mannitol therapy [192]. I believe these cases were likely due to volume depletion, with resultant renal vasoconstriction and increased susceptibility of the kidney to reduced blood pressure or other insults that compromise renal perfusion.

Dr. Madias: Is it known whether the levels of antioxidant enzymes change with ischemia?

Dr. Bonventre: It has been reported that cell glutathione levels fall with ischemia [188, 189]. Glutathione detoxifies hydrogen peroxide via glutathione peroxidase. In contrast, Yoshioka and colleagues have demonstrated that ischemia and reperfusion augments glomerular glutathione, superoxide dismutase, and catalase 6 days after the ischemic insult [178]. Mechanisms responsible for the induction of synthesis of antioxidant enzymes are not understood. This finding in the kidney is similar to the increased production of antioxidant enzymes in the lung after exposure to high oxygen tension or endotoxin [193, 194].

Dr. Michael Linshaw (*Chief, Division of Pediatric Nephrology, The Floating Hospital for Infants and Children, New England Medical Center*): Is the cause for salt wasting that occurs during the recovery phase a change in the polarity of the cell and a redistribution of the ATPase?

Dr. Bonventre: Acute renal failure is associated with an impaired ability to reabsorb sodium and water, as reflected by increases in fractional excretion of sodium and decreased concentrating ability. Even with mild ischemic insults in experimental animals, there is loss of apical and basolateral cell polarity with apical membrane localization of the Na^+-K^+ ATPase, an enzyme that normally is confined to the basolateral membrane [24, 195]. It is possible that during the recovery phase the regenerating epithelium contains cells that are not fully differentiated and polarized thus contributing to sodium wasting.

Dr. Klemens Myer (*Division of Nephrology, New England Medical Center*): Do I infer correctly that the observation of apoptosis is still qualitative?

Dr. Bonventre: Correct. There's been no detailed quantitative morphometric analysis of the extent of apoptosis present in different clinical conditions of acute renal failure.

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