MECHANISMS OF INJURY IN RENAL DISEASE AND TOXICITY

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Chapter 2

Cellular and Molecular Mechanisms of Ischemic Acute Renal Failure and Repair

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INTRODUCTION

The pathophysiology of ischemic acute renal failure is complex. Inadequate blood flow to the renal tubule cells may result from hypotension, decreased cardiac function, renal artery stenosis or occlusion, or intrarenal smaller vessel disease due to atherosclerosis, atheroemboli, or vasculitis. Furthermore, ischemia may result from local vasoconstriction due to an imbalance between vasoconstrictive and vasodilatory factors. These changes in vasoconstrictive forces may result from systemic vasoactive agents which are active locally on the small vessels of the kidney or may result from damage to the endothelium, or alteration in endothelial function resulting in decreased production of vasodilatory substances such as nitric oxide. The renal vasculature is quite sensitive to endothelin, which reduces renal blood flow and glomerular filtration rate (GFR) (Kellerman and Bogusky, 1992). (Kon, and colleagues, (Kon, et al., 1989) have found that injection of antibodies against endothelin into a branch of the rat renal artery resulted in preservation of postischemic glomerular blood flow and single nephron GFR in the region of the kidney supplied by the branch artery. In addition, vasodilatory responses to agents such as acetylcholine may be decreased in the postischemic vessel (Malis, et al., 1991; Conger, et al. 1991). Alterations in endothelial cell function may be important in local loss of autoregulation that occurs with ischemic renal failure (Conger, et al., 1988).

Heterogeneity of blood flow plays an important role in the pathophysiology of ischemic renal failure. Differences in the distribution of blood flow likely plays an important role in the heterogeneous nature of the tubule lesions seen with ischemia (Oliver, et al., 1951). Vascular congestion in the outer medulla (Mason, et al., 1989) and decreased blood flow (Vetterlein, et al., 1986) to this region is a feature of experimental ischemic acute renal failure in rats. This vascular congestion may be the result at least in part of swelling of endothelial or tubular epithelial cells, resulting in interference with flow through the vasa recta and further compromise of blood flow.

The vulnerability of the tubules in the outer medulla is also enhanced by the vasa recta countercurrent exchange of oxygen which results in a marked drop-off in oxygen tension with increasing distance into the medulla from the cortex (Leichtweiss, et al., 1969). While many medullary cells increase their rates of glycolysis in an anaerobic environment, the thick ascending limb cells in the outer medulla are not able to preserve their ATP levels despite enhanced glycolysis (Uchida and Endou, 1988). Epstein, Brezis and Rosen have published a number of reports describing the vulnerability of the thick ascending limb to structural damage in the isolated perfused kidney. They attribute this vulnerability to the imbalance in metabolic demand of this nephron segment and oxygen delivery to these cells (Brezis, et al., 1984; Epstein, et al., 1989; Rosen and Brezis, 1990). However, after 30 min of ischemia and 24 h of reflow to the kidney *in vivo*, the damage is primarily in the S3 segment of the proximal tubule (Venkatachalam, et al., 1978).

TUBULAR CELL DAMAGE

GENERAL CONSIDERATIONS

This chapter will focus on the cellular and molecular consequences of ischemia in the kidney. The result of generalized and/or localized ischemia to renal tissue is damage to the tubular cells themselves. Damaged tubular cells swell in size due to abnormalities in volume regulation. Membrane blebs are formed which break off and are released into the tubular lumen. These blebs, together with other released "cellular debris", then result in cast formation (Bayati, et al., 1989), tubular lumen obstruction, and elevated tubular pressures (Arendshorst, et al., 1975; Mason, et al., 1977). The damaged cells sluff leaving a denuded basement membrane. The resultant enhanced tubular fluid backleak into the peritubular capillaries results in an effective reduction in GFR which is likely much more important than glomerular endothelial and epithelial cell changes and resultant effects on the glomerular capillary ultrafiltration coefficient (Daugharty, et al., 1974; Hostetter and Brenner, 1988).

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CELL VOLUME REGULATION

Inhibition of cellular metabolism results in depletion of ATP and loss of the energy reserve necessary to energize membrane transporters which drive transmembrane electrolyte movement and maintain the internal electrolyte content of the cell. Since volume regulation is tightly coupled to an unequal distribution of electrolytes across the cell membrane maintained by these active transporters, it is not surprising that there is dysregulation of cell volume with ischemia. Cells swell with ischemia. While this swelling may have consequences for the integrity of the tubule lumen itself, perhaps the most important consequences occur as a result of the effect of swelling on the tubules' vascular supply, in particular in the outer medulla. Tubular cell swelling in the outer medulla can obstruct the peritubular capillaries, resulting in medullary congestion, decreased medullary blood flow, and further ischemia and tubular cell injury, setting up a positive feedback process enhancing the injury.

Cell volume is maintained at least in part due to the action of the Na^+,K^+-ATPase pump which maintains an intracellular electrical negativity and low levels of intracellular Na^+ concentration (Flores, et al., 1972; Leaf, 1959). With oxygen deprivation and ATP depletion the Na^+,K^+-ATPase pump becomes inactive, the cells become depolarized, and intracellular Na^+ and Cl^- accumulate. As a result the cells take up water and swell. An intact cytoskeleton appears to play a role in maintenance of normal proximal cell volume (Linshaw, et al., 1990). Disruption of the cytoskeleton as a consequence of ischemia may contribute to the cell swelling seen. Addition of impermeant solutes have been added to renal preservation solutions in order to prevent this cell swelling (Bonventre and Weinberg, 1992).

Mason and colleagues used electron microprobe techniques to measure total cellular Na^+ and Cl^- in proximal and distal tubules after ischemia in rats (Mason, et al., 1981). They found increased Na^+ and Cl^- content in both proximal and distal cells, with the largest changes in proximal cells. Ischemia-related changes in electrolyte composition and cell volume are most pronounced in the S3 segment of the proximal tubule (Beck, et al., 1992).

Cell swelling has very different consequences in an isolated cell when compared to an organ. Although preservation of cell volume may not prevent cell death in an isolated cell preparation, in the organ prevention of cell swelling may prevent tubular and venous obstruction and allow more oxygen delivery to the partially ischemic or postischemic tissue. Agents which inhibit cell swelling, such as mannitol, partially protect the kidney against the functional deficits induced by ischemia and decrease the amount of outer medullary congestion (Malis, et al., 1983; Mason, et al., 1989).

CELL POLARITY

The earliest morphological changes that occur with ischemia include loss of the apical brush border and blebbing of apical membranes. Molitoris and colleagues (Canfield, et al., 1991; Molitoris, 1991; Molitoris, et al., 1989; Molitoris, et al., 1988) have found that after 10 min of ischemia, Na^+,K^+-ATPase activity, normally present only on the basolateral aspects of proximal tubular cells, is found on the apical membrane also. In addition, there are marked changes in apical membrane lipid composition after periods of ischemia as short as 5 min. These changes, together with the loss of basolateral interdigitations (Jones, 1988), likely explain the functional decrease in Na^+ and Na^+-coupled proximal transport properties. Changes in the actin cytoskeleton may be responsible for the loss of polarity of the proximal tubule cell (Kellerman, et al., 1990). There is disruption and redistribution of the cortical actin cytoskeleton (Kellerman, et al., 1990) and a large percentage of F-actin loss occurs within the first 5 min of ischemia (Kellerman and Bogusky, 1992).

The redistribution of Na^+,K^+-ATPase from basolateral to apical membrane after 15 min of ischemia was specific to the proximal tubule and was not seen in distal convoluted tubules or thick ascending limbs. With ischemia the Na^+,K^+-ATPase was released from cytoskeletal attachments (Molitoris, et al., 1992) and it has been hypothesized that with ischemia the enzyme moves within the lipid bilayer of the cell from basolateral to apical membranes facilitated by release from cytoskeletal attachments and opening of the tight junction (Molitoris, et al., 1989). While there is good evidence that these changes in polarity occur with short periods of ischemia and these alterations in cell polarity likely affect transepithelial vectorial transport, it is not clear how these changes contribute to the marked reductions in GFR characteristic of the postischemic kidney.

ADENINE NUCLEOTIDE METABOLISM

Cellular levels of ATP fall with oxygen deprivation in tubular cells that normally generate their energy currency as a result of aerobic metabolism. With prolonged ischemia mitochondria swell and "flocculent

mitochondrial densities" appear. Ultimately the mitochondria become so dysfunctional that even if oxygen is restored they cannot generate ATP. Complete ischemia of the kidney for 10 min results in a 70 to 90% reduction in ATP levels (Gerlach, et al., 1963). Cortical levels drop much more dramatically than medullary levels (Jones and Welt, 1967; Kessler, 1970), reflecting the glycolytic metabolism of some medullary cells, which are better adapted to oxygen deprivation which is a characteristic of the environment that they normally function in even when blood flow to the kidney is normal. In the outer medullary collecting duct glycolytic metabolism alone can maintain ATP levels at 60 to 70% of normal (Stokes, et al., 1987; Uchida and Endou, 1988; Zeidel, et al., 1986) and can sustain acid secretion (Zeidel, et al., 1986). When oxidative metabolism is blocked, these nephron segments can also increase their glycolytic rate in a much more efficient way than can the proximal tubule cells (Bagnasco, et al., 1985). Not all medullary cells have this capacity to maintain ATP levels to a great extent with glycolysis. The medullary thick ascending limb, for example, is not able to sustain normal ATP levels in the face of inhibition of oxidative metabolism despite its enhanced glycolytic rate (Uchida and Endou, 1988). Proximal tubule cells also have a limited capacity to generate ATP via glycolysis (Bastin, et al., 1987).

Many cellular processes are critically dependent upon ATP hydrolysis. These processes cease or become markedly impaired when cellular ATP is markedly depleted. Ion gradients will dissipate without the ATP necessary for the ion transporter ATPases involved in maintenance of the ionic gradients. Sodium and calcium may accumulate in the cell. Deacylation, acylation cycling will be disrupted and fatty acids will accumulate due to the absence of energy required for the reacylation. Acidosis will develop as a consequence of increased glycolytic metabolism.

When ATP is degraded, ADP and AMP are formed. AMP is then acted upon by 5′-nucleotidase, or is first converted to IMP by adenylate deaminase followed by 5′-nucleotidase to form adenosine, inosine, and hypoxanthine. Although the nucleotides (ATP, ADP, AMP, IMP) have low permeability to the cell membrane, the nucleosides, adenosine and inosine, and the base, hypoxanthine, are permeable and can readily diffuse out of cells. Furthermore, the hypoxanthine can be converted by xanthine oxidase to uric acid. Thus, as a result of either diffusion out of the cells or metabolism there is a decrease in the purine substrate pool.

During the reoxygenation period ATP reaccumulates. The extent or rate of accumulation may be a critical factor in cell survival. In the postischemic kidney ATP is synthesized primarily via "salvage pathways" from AMP, adenosine, adenine, or other purine bases, nucleosides or nucleotides. Thus, loss of these precursors will severely limit the extent of ATP production.

If ATP levels per se are important determinants of recovery of kidney function after an ischemic insult, then maneuvers designed to enhance ATP levels should be protective. Siegel and colleagues (Siegel and Gaudio, 1988; Siegel, et al., 1980) demonstrated that intravenous infusion of ATP and $MgCl_2$ protected kidneys against 30 min of ischemia, even when administered after the ischemic insult. This protection was associated with increased rate of recovery of ATP levels (Stromski, et al., 1986). Since AMP was as effective as ATP it was suggested that the protective effect was due to an increase in the nucleotide precursor pool available for the resynthesis of ATP. Consistent with this conclusion, AMPCP, which inhibits 5′-nucleotidase (Van Waarde, et al., 1989), also protected kidneys against ischemic injury.

CALCIUM

Calcium is critical to cellular function. It is involved in the regulation of contraction, mobility, secretion, membrane stability, cell division, phagocytosis, and many enzymatic processes. It has been proposed that increased levels of cellular calcium concentration are directly related to the cell injury due to ischemia. While it is clear that damaged cells accumulate calcium, it is less clear whether or not an increase in cellular calcium is necessary and sufficient for cell death associated with ischemia. Normally there is a large Ca^{2+} concentration gradient between the cytosolic compartment of eukaryotic cells and the extracellular environment. A 10,000-fold concentration gradient (1 m*M* extracellular to 100 n*M* intracellular free calcium concentration) is maintained by energy-dependent calcium extrusion mechanisms and low permeability of the plasma membrane to calcium. As ATP levels fall with ischemia, Ca^{2+} extrusion from the cell via Ca^{2+} ATPases (Schatzman, 1966) will be inhibited. Furthermore, the increase in cytosolic Na^+ concentration that results from decreased Na^+ extrusion via the Na^+,K^+-ATPase pump will potentiate Ca^{2+} entry into the cell via the Na^+/Ca^{2+} exchanger (Gmaj, et al., 1979; Snowdowne and Borle, 1985). Small amounts of additional cell calcium will be buffered by nonmitochondrial compartments (Cheung, et al., 1986). The mitochondria have much larger buffering capabilities, and if

large amounts of Ca^{2+} enter the cells the mitochondria can become calcium laden. Much of this uptake of calcium into the mitochondria may take place during the reperfusion period when there is an increase in cellular ATP levels, since ATP facilitates calcium uptake markedly. When mitochondria take up large amounts of Ca^{2+} they swell (Lehninger, 1970), oxidative phosphorylation becomes uncoupled (Rossi and Lehninger, 1964), and free fatty acids are released.

An increase in cytosolic free Ca^{2+} concentration will activate Ca^{2+}-dependent proteases and phospholipases which leads to proteolysis and membrane disruption (see below). Elevated Ca^{2+} levels may also disrupt the cell cytoskeleton (Yin and Stossel, 1979).

If Ca^{2+} is important for cell death associated with ischemia it might be expected that cellular $[Ca^{2+}]$ would increase prior to the initiation of irreversible cell damage. In isolated cardiac myocytes exposed to 30 min of anoxia and substrate deprivation, we found that cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and total cellular and mitochondrial Ca^{2+} content did not increase, despite evidence for irreversible contracture and mitochondrial dysfunction (Cheung, et al., 1986; Cheung, et al., 1985; Cheung, et al., 1982). In isolated rabbit proximal tubules Weinberg found that total cell calcium did not increase until there were other indications of lethal cell injury (Weinberg, 1985). Electron probe microanalysis studies revealed that calcium overload measured in tubules was due to calcium-overloaded mitochondria in already lethalty damaged cells (Lefurgey, et al., 1986). Other investigators have measured increases in $[Ca^{2+}]_i$ with anoxia in renal tubules or renal cells in culture exposed to inhibitors of metabolism (chemical anoxia). Small changes in $[Ca^{2+}]_i$ have been measured in anoxic isolated cardiac cells (Snowdowne, et al., 1985a) or cultured kidney cells (Snowdowne, et al., 1985b). Mandel and colleagues (Mandel, et al., 1987) reported that $[Ca^{2+}]_i$ was unchanged after 40 min of anoxia, at a time when there was impaired respiration and plasma membrane damage in kidney tubules (Jacobs, et al., 1991). Under these conditions there was insufficient ATP to support mitochondrial uptake of calcium. In liver cells exposed to conditions which decrease ATP levels, changes in $[Ca^{2+}]_i$ do not precede loss of cell membrane integrity and mitochondrial dysfunction (Lemasters, et al., 1987). Some investigators report changes in $[Ca^{2+}]_i$ that are much smaller than those observed acutely with physiological processes such as cell fertilization or contraction (10- to 25-fold [Poenie, et al., 1986; Poenie, et al., 1985]), hormonal stimulation (8-fold [Bonventre, et al., 1986]), or extracellular Na^+ depletion (12-fold [Snowdowne and Borle, 1985]).

If entry of calcium from the extracellular milieu is responsible for cell death with ischemia, then it follows that removal of extracellular calcium might be protective and increases in extracellular calcium might exacerbate the injury. Wilson and Schrier (1986), using vital dye exclusion as a measure of viability, found that viability of proximal tubular cells in primary culture that were exposed to anoxia was greater at 48 h if Ca^{2+} had been removed from the media for the initial 2-h postanoxia. Takano et al. (1985) found that exposure of isolated renal tubules to 30 min of anoxia in the presence of a low $[Ca^{2+}]$ medium (2 μM) resulted in a reduced LDH release during the anoxic period, but an enhanced release during reoxygenation. Interpretation of studies in which extracellular calcium concentration is reduced is hampered by the independent detrimental effects of removal of extracellular calcium on cell function. 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 (Cheung, et al., 1982). Smith and colleagues (1981) found that hepatocytes were more susceptible to toxic injury in the absence of Ca^{2+} than in its presence. In experiments designed to define an optimal concentration of calcium for the perfusate in order to optimize kidney preservation, it was reported that 0.5 mM $[Ca^{2+}]$ was optimal with higher and lower concentrations resulting in more organ failure (McAnulty, et al., 1989).

With the goal of specifically blocking calcium uptake without reducing extracellular $[Ca^{2+}]$, Ca^{2+} channel blockers have been used to explore the role of Ca^{2+} in ischemic cell injury. There are studies *in vivo* which demonstrate efficacy of these agents to protect against ischemic injury (Burke, et al., 1984; Goldfarb, et al., 1983). Protection is associated with less calcium loading of mitochondria and preservation of mitochondrial function (Widener and Mela-Riker, 1984). There has been some controversy over the interpretation of these results. Some have concluded that the effects are due to direct action of the pharmacologic agents to prevent calcium from entering the epithelial cell (Burke, et al., 1984). In a model of fixed ischemia *in vivo* and in the isolated rat kidney, under conditions where verapamil did not alter renal blood flow, we found no protection with this agent (Malis, et al., 1983). Verapamil did protect against ischemic injury in a norepinephrine model of acute renal failure. We concluded that the protection was due to an inhibition of norepinephrine-induced renal vasoconstriction.

Consistent with the conclusion that calcium channel-blocking agents exert their protective effects primarily at the level of the vasculature is the observation that marginal effects of high doses of verapamil are observed when isolated kidney tubules are exposed to anoxia (Weinberg, et al., 1984).

We found that verapamil and nifedipine did not protect isolated non-contracting heart cells (Cheung, et al., 1984) or kidney tubules (unpublished) against injury associated with 30 to 40 min of anoxia. When anoxic cardiac myocytes were paced to contract, verapamil and nifedipine protected by decreasing the cellular contractile activity (Cheung, et al., 1984). Ca^{2+} channel blockers have been used to try to decrease acute renal failure in the renal allograft. They have been administered to both donor and recipient with resultant reduction in the incidence of acute renal failure accounting for delayed graft function. It is possible that these agents are working on the vasculature to enhance blood flow to the renal tubular cells. The recipients also received cyclosporine, which results in vasoconstriction. Thus the calcium channel blocker may have blocked the vasoconstriction that is likely responsible, at least in part, for cyclosporine toxicity (Dawidson and Rooth, 1990; Wagner, et al., 1987).

Ca^{2+} channel-blocking agents have other effects which also complicate the interpretation of their actions. They antagonize calmodulin and phosphodiesterases (Bostrom, et al., 1981; Epstein, et al., 1982). They also may have nonspecific "local anesthetic" or "membrane-stabilizing" actions due to interaction with hydrophobic regions of the membrane bilayer (Katz, 1985).

In summary, the precise role played by calcium in the pathophysiology of acute tubular cell injury remains unclear. There is evidence that increases in intracellular $[Ca^{2+}]$ are neither necessary nor sufficient to explain irreversible cell injury. Calcium may nevertheless be important. Increases in cytosolic $[Ca^{2+}]$ may be permissive. For example, uptake of amounts of calcium into mitochondria, which of itself is not injurious, predisposes the mitochondria to damage induced by reactive oxygen species (ROS) (Malis and Bonventre, 1986). Furthermore, enzymes that participate in the cell injury may be modified so as to be more sensitive to Ca^{2+}. If the enzyme is more "calcium sensitive" then Ca^{2+} may play an important role in the injury mechanism without there being any significant increase in cytosolic $[Ca^{2+}]$. We have found, for example, that the Ca^{2+} sensitivity of phospholipase A_2 (PLA_2) in isolated neurons *in vitro* is enhanced by glutamate, under conditions that mimic brain ischemia (Kim, et al., submitted).

ACIDOSIS

Kidney ischemia stimulates glycolysis, thus increasing the generation of protons and leading to a decrease in cellular pH (Chan, et al., 1982). Severe acidosis may be detrimental to cell survival in other organs, such as the heart (Williamson, et al., 1976; Neely and Grotyohann, 1984). The reduced pH inhibits glycolysis by inhibiting phosphofructokinase, the rate-limiting enzyme in glycolysis. Although severe acidosis has been proposed to be detrimental to the kidney (Bore, et al., 1981) we have demonstrated in renal tubular cells and isolated hepatocytes that mild acidosis (pH 6.9) protects cells against anoxia and substrate deprivation *in vitro* (Bonventre, 1984; Bonventre and Cheung, 1985). Weinberg also found protection of renal tubular cells with acidosis (Weinberg, 1985). This protection is associated with the prevention of any increase in total cell Ca^{2+} (Bonventre and Cheung, 1985). When tubules were incubated at high density and generated an extracellular pH of approximately 7.0 due to accumulation of products of metabolism, similar protection was observed (Weinberg, 1985). Acidosis was also found to be protective in the isolated perfused kidney (Shanley, et al., 1988). The mechanism of protection by mild degrees of acidosis is not known. The protection is not dependent upon preservation of cellular ATP levels (Bonventre and Cheung, 1985). Reduction in pH protects tubules exposed to ionomycin or mitochondrial inhibitors despite the fact that the tubules have increases in $[Ca^{2+}]_i$ equivalent to those changes observed in nonprotected tubules (Weinberg, et al., 1991). Acidosis has been reported to stabilize cell membranes (Bell, et al., 1971) and may decrease transmembrane calcium flux (Vogel and Sperelakis, 1977). It is possible that the protection is due to inhibition of an enzymatic process which would otherwise be detrimental to the cells. For example, we have found that at least two kidney PLA_2 enzymes have alkaline pH optima (Bonventre, 1992; Nakamura, et al., 1991). When blood flow and oxygen delivery are reestablished the extracellular pH rapidly returns to normal. With the increase in intracellular pH upon reperfusion, activation of PLA_2 would be enhanced. This might lead to further alteration in membrane structure and secondarily to enhanced Ca^{2+} permeability, which in turn will further enhance PLA_2 activation. The increase in pH which occurs as a consequence of reperfusion may contribute in an important way to "reperfusion injury".

REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) have been implicated in various forms of cellular injury, including ischemic insults (McCord, 1985; Halliwell, 1987). There are several kinds of evidence implicating ROS in ischemia-reperfusion injury. Products of lipid peroxidation, such as malondialdehyde and ethane, have been detected with ischemia-reperfusion (Paller and Hebbel, 1986; Paller, et al., 1984). Various ROS scavengers, such as superoxide dismutase, DMSO, and dimethylthiourea, afford protection against ischemic injury (Paller, et al., 1984; Kedar, et al., 1983).

There are a number of sources of ROS in the postischemic tissue. One source of ROS is xanthine oxidase. Tissue xanthine oxidase levels are increased due to conversion from xanthine dehydrogenase with ischemia. Under normoxic conditions xanthine dehydrogenase catalyzes the transfer of electrons to NAD as xanthine and hypoxanthine are oxidized to uric acid. This enzyme is converted to xanthine oxidase with ischemia and reperfusion by a Ca^{2+} dependent protease (McCord, 1985). Xanthine oxidase uses molecular oxygen as an electron acceptor and generates superoxide during the oxidation of hypoxanthine, which is increased in concentration in the postischemic tissue as discussed above. In one study, tissue hypoxanthine levels were increased approximately 300-fold after 60 min of renal ischemia (Osswald, et al., 1977). The mitochondria constitute a second source of ROS. When mitochondria are deprived of oxygen the electron transport chain intermediates become more reduced and free electrons may result in enhanced superoxide generation when oxygen delivery to the tissue is restored. A third source of ROS are the enzymes involved in the metabolism of arachidonic acid, PGH synthase, and lipoxygenase. Both of these enzymes produce superoxide in the presence of NADH or NADPH (Kukreja, et al., 1986).

A fourth source of ROS are the neutrophils present in the postischemic tissue. These neutrophils can directly participate in the damage to the epithelial or endothelial cell (Rubanyi and Vanhautte, 1986a, 1986b) or may act via the production of cytokines, or leukotrienes, which may constrict the vessels, further compromising local blood supply (Hellberg and Kallskog, 1988; Klausner, et al., 19[illegible]). Activated neutrophils produce superoxide as a result of activity of the NADPH oxidase enzyme (Rossi, 1986). Neutrophils have been shown to accumulate in the kidney following ischemia and reperfusion (Hellberg and Kallskog, 1988). Neutropenia protects the kidney against ischemia-reperfusion injury in some studies (Hellberg and Kallskog, 1988; Klausner, et al., 1989), but not in others (Paller, 1989; Thornton, et al., 1989). In addition, reperfusion of isolated perfused kidneys with activated neutrophils can accentuate renal functional impairment observed with ischemia (Linas, et al., 1988; Linas, et al., 1992). A fifth source of ROS is the autooxidation of catecholamines, which results in the generation of superoxide and hydrogen peroxide (Bors, et al., 1978; Misra and Fridovich, 1972). Finally, the oxidative enzymes of the endoplasmic reticulum, such as those of the P-450 mixed-function oxidase system and the peroxisomes, represent other potential sources of ROS.

There are a number of ways in which tissue damage can result from ROS. ROS can cause lipid peroxidation through removal of a hydrogen atom from a polyunsaturated fatty acid of membrane phospholipids. The conjugated diene which then forms can react with oxygen to form a peroxide radical which can remove hydrogen atoms from other lipids, generating a chain reaction. This process of lipid peroxidation will increase plasma and subcellular membrane permeability (Kappus, 1985). Peroxidized lipids have increased susceptibility to degradation by PLA_2 (Weglicki, et al., 1984; Savanian, et al., 1981; Savanian and Kin, 1985). ROS alter the function of the Na^+,K^+-ATPase pump (Kako, et al., 1988) and damage DNA (Brawn and Fridovich, 1985; Weitberg, et al., 1985). ROS may directly oxidize cellular proteins (Fliss, 1988) involved in critical cellular functions, including those involved in calcium extrusion mechanisms.

Despite a large amount of investigation, the role of ROS in ischemic kidney injury remains somewhat controversial. Not all investigators agree on protective effects of antioxidants (Greene and Paller, 1991) nor do all agree on the presence of enhanced lipid peroxidation or ROS generation with ischemia (Gamelin and Zager, 1988; Greene and Paller, 1991). Some have reported protection with exogenously administered superoxide dismutase, or catalase. Allopurinol, which has been used to inhibit xanthine oxidase, has also had mixed success as a protective agent (Borkan and Schwartz, 1989). Furthermore, the human kidney likely has very little xanthine oxidase (Southard, et al., 1987). Glutathione, normally present in high amounts in tubular cells, can neutralize ROS. Cellular glutathione levels fall with hypoxia and reduced cellular glutathione levels sensitize cells to oxidative stress (Arrick, et al., 1982). As with other ROS scavengers, results with glutathione are conflicting (Paller, 1988; Yang, et al., 1990). It has

been argued that protective effects of this compound are due to the generation of glycine, its metabolic product, which is protective independent of any effect on ROS (Weinberg, 1991).

PHOSPHOLIPASES

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. There is an increasing number of members of this family, and how each one is involved in various aspects of cell function is the topic of active research in a number of laboratories (Bonventre, 1992). PLA_2 likely plays an important role in ischemic cell injury (Bonventre, 1988; Bonventre, et al., 1988). Phospholipid degradation contributes to ischemic tissue injury in the kidney (Spencer, 1976), brain (Bazan, 1970), heart (Chiariello, et al., 1987), intestine (Otamiri and Tagesson, 1989), and liver (Farber, et al., 1981). PLA_2 can directly disrupt membrane integrity. Furthermore, ATP depletion may inhibit reacylation of lysophospholipids (Chien, et al., 1984). In addition, the products of phospholipid degradation, lysophospholipids and free fatty acids, can alter membrane permeability characteristics and uncouple mitochondrial respiration. The eicosanoid products of arachidonic acid metabolism are chemotactic for neutrophils which may contribute to the tissue injury. In addition, when 1-*O*-alkyl-2-acylphosphorylcholine is the substrate for PLA_2 the product becomes the precursor for platelet activating factor, which may activate platelets, resulting in capillary occlusion due to platelet plugging.

Mitochondria represent particularly important sites of PLA_2 action (Malis and Bonventre, 1986; Nishida, et al., 1987). Changes in mitochondrial membrane integrity impair ATP generation (Spencer, 1976). We identified a likely role for PLA_2 in mitochondrial injury associated with Ca^{2+} and ROS. We evaluated *in vitro* the damage produced in isolated rat renal cortical mitochondria after exposure to Ca^{2+} or ROS alone, and compared the mitochondrial damage to that evoked by the combination of Ca^{2+} and ROS (Figure 1). ROS were generated by hypoxanthine, xanthine oxidase, and iron (Malis and Bonventre, 1986). Mitochondria exposed to ROS alone sustained an increase in substrate-supported (state 4) respiration due to increased mitochondrial membrane permeability, a decreased ADP-stimulated (state 3) respiration, and a 50% reduction in the respiratory control ratio (state 3/state 4). F_1F_0 ATPase was inhibited, but there was no damage to the electron transport chain or ATP-ADP translocase. In contrast, after exposure of mitochondria to a small amount of Ca^{2+} (30 nmol/mg mitochondrial protein), which by itself did not change mitochondrial function, addition of ROS resulted in a marked enhancement of mitochondrial membrane permeability, a major functional defect in the electron transport chain, marked decrease in F_1F_0 ATPase and adenine nucleotide translocase activity, and complete uncoupling of oxidative phosphorylation. PLA_2 was at least partially responsible for this synergistic interaction between Ca^{2+} and ROS. Dibucaine, a PLA_2 inhibitor, partially protected the electron transport chain, F_1F_0 ATPase, and adenine nucleotide translocase from damage.

Since lipid peroxidation of cell membranes predisposes to degradation by PLA_2 and since membranes that are enriched with polyunsaturated fatty acids are more susceptible to lipid peroxidation, we predicted that mitochondria enriched with polyunsaturated fatty acids would have enhanced susceptibility to damage induced by Ca^{2+} and ROS. This was the case when mitochondrial injury was compared in organelles taken from fish oil-fed rats with those taken from animals fed normal diets or diets rich in beef tallow. The greater damage in the mitochondria from fish oil-fed rats was associated with enhanced PLA_2 activity and could be prevented in part by PLA_2 inhibitors (Malis, et al., 1990).

The contribution of PLA_2 to intact cell injury associated with ischemia and energy deprivation has been controversial (Bonventre, 1988; Van der Vusse, et al., 1989). PLA_2 treatment of hypoxic proximal tubules results in severe cellular injury and significant decreases in uncoupled respiratory rates of the tubules, reflective of electron transport chain damage (Nguyen, et al., 1988). It is likely, however, that much of the involvement of PLA_2 in the injury process is related to activation of intracellular forms of the enzyme. Much of the evidence supporting a role for PLA_2 in ischemia has been indirect, based upon measured increases in free fatty acids in postischemic tissues or protection afforded by PLA_2 inhibitors that are not very selective. The role of Ca^{2+} in activation of PLA_2 also has been unclear, since many forms of PLA_2 require very high Ca^{2+} concentrations for activation, and the $[Ca^{2+}]$ sensitivities of intracellular forms of PLA_2 have not been 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 (Venkatachalam, et al., 1988). The levels to which free fatty acids accumulated correlated very well with cell injury. There is evidence in other organ systems that PLA_2 is involved in ischemic injury. PLA_2 inhibitors (albeit not specific) afford protection against phospholipid

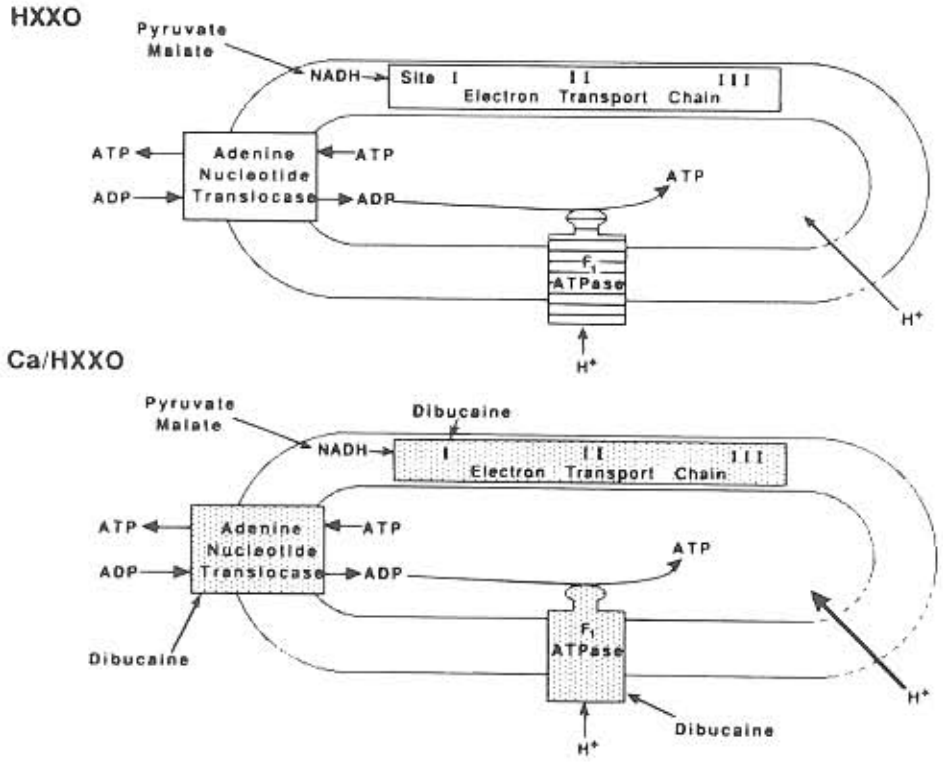

Figure 1 Summary of mitochondrial injury induced by reactive oxygen species alone or calcium and reactive oxygen species. Reactive oxygen species were generated by hypoxanthine (HX) and xanthine oxidase (XO). With exposure of isolated kidney mitochondria to HX and XO (upper panel) there was partial inhibition of the F_1-ATPase (indicated graphically by light hatching of the icon representing the ATPase) and increase in the membrane permeability (indicated graphically by breaks in the membrane) allowing passive backleak of H^+ ions. In the lower half of the figure is depicted the damage induced by HX and XO to mitochondria previously loaded with an amount of $CaCl_2$ (30 nmol/mg mitochondrial protein) which alone causes no mitochondrial damage. Under these conditions, damage to the mitochondria is severe with: (1) marked inhibition of the electron transport chain due to damage to Site 1; (2) inhibition of the adenine nucleotide translocase; (3) reduction in the function of the ATPase; and (4) marked increase in the mitochondrial membrane permeability to H^+. Dibucaine partially protects Site 1, the adenine nucleotide translocase and the F_1-ATPase, suggesting that PLA_2 activation is responsible, at least in part, for these components of mitochondrial damage.

degradation, lead to a reduction in infarct size after coronary artery occlusion in rats (Chiariello, et al., 1987), and also partially preserve the mucosal permeability barrier in the ischemic and reperfused intestine (Otamiri and Tagesson, 1989). An inhibitor of PLA_2 activity and lipid peroxidation protects the brain against injury caused by transient global forebrain ischemia (Clemens, et al., 1991). Data with PLA_2 inhibitors are frequently non-conclusive, however, because many of the inhibitors are nonspecific and have different effects on different forms of the enzyme.

As an initial attempt to understand the role of PLA_2 in ischemic injury, we characterized PLA_2 activities in cytosolic, mitochondrial, and microsomal fractions of rat kidneys (Nakamura, et al., 1991). Two forms of PLA_2 activity were present in the cytosolic fraction. A high-molecular weight form, active against phosphatidylcholine (PC) and phosphatidylethanolamine (PE), has been purified and identified to have a molecular mass of approximately 100 kDa by gel electrophoresis (Gronich, et al., 1990). A smaller form with an M_r of approximately 14 kDa, active against PE, was also found in the cytosolic fraction. In the mitochondrial and microsomal fractions a single form ($M_r \approx 14$ kDa) was dominant, active against both PC and PE.

The PLA_2 activities of cytosolic, mitochondrial, and microsomal extracts of rat kidney were measured after ischemia and reperfusion and compared with the activities of control tissue extracts (Figure 2). Each of the extracts was assayed under identical free $[Ca^{2+}]$ and at pH 7.5. Cytosolic, mitochondrial,

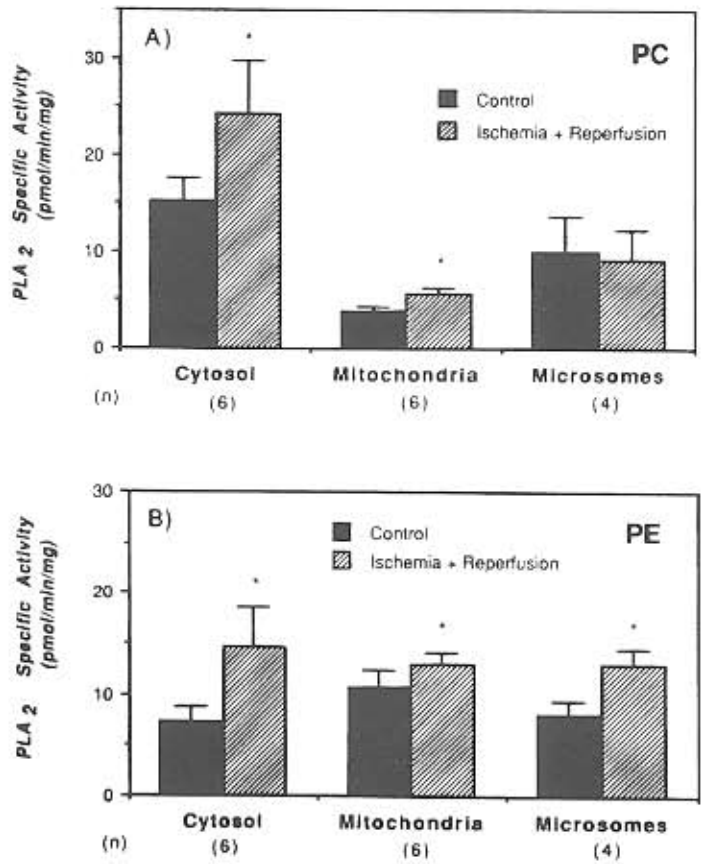

Figure 2 Cytosolic, mitochondrial, and microsomal PLA_2 (A and B) activities in control (contralateral kidney) extracts and extracts of kidneys removed after 45 min of ischemia and 1 h of reperfusion. Cytosolic and mitochondrial PLA_2 activities were increased in the post-ischemic kidney extracts when PC was the substrate (A). Cytosolic, mitochondrial, and microsomal extract PLA_2 activities were increased when PE was the substrate (B). *$p < .05$ compared to control. (From Nakamura, et al. (1991) *J. Clin. Invest.* 87: 1810–1818 With permission.)

and microsomal activities were enhanced in kidneys exposed to 45 min of clamp-induced ischemia with 1 h of reperfusion when PE was used as substrate.

In the rat kidney, cytosolic PLA_2 activity was enhanced when $[Ca^{2+}]$ was increased from 100 n*M*, a value representing baseline cytosolic $[Ca^{2+}]$ in renal epithelial cells, to 200 to 300 n*M*, values well within the range reached in response to both physiological and pathophysiological stimuli (Bonventre and Swidler, 1988). Mitochondrial PLA_2 activity was also $[Ca^{2+}]$ dependent, but this activity required higher $[Ca^{2+}]$. Activity increased, however, as $[Ca^{2+}]$ increased from 1 to 5 μM, values within the range of variation of mitochondrial matrix $[Ca^{2+}]$ under physiological and pathophysiological states. While it is thus clear that PLA_2 activity could be expected to increase with increasing cytosolic $[Ca^{2+}]$, this is not the explanation for the increase observed with ischemia and reperfusion, since the assay is performed with a fixed concentration of calcium *in vitro*. Thus ischemia and reperfusion results in stable activation of both soluble and membrane-associated forms. This stable activation of PLA_2 may play a major role in cellular injury associated with ischemia and reperfusion.

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 have reported and discussed above (Bonventre and Cheung, 1985).

There are likely additional forms of PLA_2 present in the kidney. Morrison and Irwin (1990) have reported in an abstract the presence of a Ca^{2+}-dependent, PE-specific cytosolic PLA_2 in the rabbit renal cortex. Portila and colleagues (1992) reported a 40-kDa form, immunologically related to the pancreatic Group I PLA_2 which is increased in activity with exposure of rabbit renal proximal tubules to anoxia.

In the heart a Ca^{2+}-independent, plasmalogen-specific form of the enzyme has been described which is activated by ischemia (Ford, et al., 1991). While such forms have not been identified in the kidney, it is clear that were one present it would likely be important since the kidney 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 PE. This preferential incorporation is greater in kidney than in heart or liver (Arthur and Page, 1991).

While elevated cytosolic [Ca^{2+}] may be important for activation of the PLA_2 enzymes *in vivo* with ischemia, the preservation of enhanced activity *in vitro* under conditions of our assay, where [Ca^{2+}] is fixed, suggests a stable modification of the enzymes. Thus ischemia and reperfusion may result in a covalent modification of the enzyme and PLA_2 activation may prevail even if [Ca^{2+}] levels return to baseline values *in vivo* after ischemia. This may contribute to the accumulation of arachidonic acid and metabolic products of arachidonic acid in tissues after reperfusion (Nakano, et al., 1990).

PLA_2 may be regulated via phosphorylation by protein kinase C (PKC) or MAP kinase (Bonventre and Swidler, 1988; Lin, et al., 1993; Nemenoff, et al., 1993). There is evidence that PKC is activated during ischemia in the brain (Onodera, et al., 1989). Phosphorylation of the enzyme may enhance its activity. We have reported in the mesangial cell that PKC activation with phorbol esters enhances the Ca^{2+} sensitivity of PLA_2 activity (Bonventre and Swidler, 1988).

In summary, multiple forms of PLA_2 are present in the cytosolic and membrane (mitochondrial and microsomal) compartments of the kidney and other organs. The [Ca^{2+}] sensitivities of both cytosolic and membrane-bound PLA_2 activities indicate that the enzymes are likely regulated by Ca^{2+} "*in vivo*". Although changes in [Ca^{2+}] may play a permissive role in these stable modifications of enzymatic activity, the changes in PLA_2 activity observed with ischemia are likely not explained by changes in [Ca^{2+}] alone. It is possible that other regulatory influences may play an important role in PLA_2 activation and mediation of cellular injury after an ischemic insult.

AMINO ACIDS

Weinberg and colleagues have found that glycine and alanine can be protective against injury due to increases in cytosolic free [Ca^{2+}], ROS, ATP depletion, and Na^+,K^+-ATPase inhibition in isolated kidney tubules and epithelial cells in culture (Weinberg, et al., 1987; Weinberg, et al., 1991a; Weinberg, et al., 1991b). Concentrations of glycine of 0.25 to 2.0 m*M* are effective. Protection of kidney cells afforded by glutathione has been attributed by Weinberg and colleagues to the formation of glycine due to glutathione degradation (Weinberg, et al., 1989, 1990, 1987). Glutathione is rapidly broken down to cysteine, glycine, and glutamate. Of these agents only glycine is protective. With ischemia *in vivo* it is unlikely that levels of glycine fall below the concentrations found to be protective; however, depletion of glycine which is permeable to cell membranes may be clinically relevant in the transplanted kidney which may become depleted of glycine during storage. The protective effects of alanine appear to be similar to those of glycine (Garza-Quintero, et al., 1990). The mechanism by which glycine and alanine protect the tubular epithelial cell remains unknown (Weinberg, 1991).

PROTEASES

Renal brush border membranes are rich in proteases (Bond and Butler, 1987). The involvement of these proteases, which include meprin, endopeptidase 24.11, and exopeptidases, or the Ca^{2+}-dependent neutral cysteine proteinases, calpains (Mellgren, 1987), in ischemic injury, is not well understood. Calpains have been implicated in a number of cellular functions, including membrane calcium channel regulation, protein kinase activity, receptor expression, and modification of cytoskeletal proteins (Belles, et al., 1988; Mellgren, 1987).

APOPTOSIS

Up until this point we have discussed injury to the cell as if it were an innocent bystander passively reacting to injurious influences which resulted in cell death either due to breakdown in its own metabolic processes or due to toxicity of exogenous influences. We have discussed one type of cell death, that is, "necrosis". It may be, however, that there are certain circumstances when the cell contributes to its own demise actively, perhaps programmed to commit suicide so that the organ would be preserved. Certain types of cell death are finely controlled by active processes. For example, during metamorphosis and embryonic development "programmed cell death" permits the proper formation of the organism. Cell death genes have been identified in the nematode, *Caenorhabditis elegans* (Yuan and Horvitz, 1990). Testosterone withdrawal results in involution of the prostate by a process involving programmed cell death (Buttyan, 1991). Abnormalities in the control mechanisms for programmed cell death may explain certain neurodegenerative diseases (Ellis, et al., 1991).

The pathological features of necrosis are very different from those of programmed cell death (Searle, et al., 1982). The latter has been called "apoptosis", a term coined by Kerr et al., who attributes the derivation of the term to Professor James Cormack of the University of Aberdeen (Kerr, et al., 1972). "Apoptosis" is a Greek term to describe "dropping off" or "falling off" of petals from a flower or leaves from a tree. Pathologically, the early stages of apoptosis are characterized by disappearance of microvilli, chromatin condensation at the periphery of the nucleus, condensation of cytosolic components, breakdown of epithelial desmosomal attachments, followed by cell surface protuberances, and fragmentation of the nucleus. Ultimately there is blebbing off of cell surface protuberances containing condensed cytosol and occasional nuclear fragments, to generate spherical or ovoid "apoptotic bodies" (Wyllie, et al., 1980) which can be phagocytosed by macrophages or epithelial cells. Apoptosis histologically is often associated biochemically with cleavage of double-stranded DNA at the linker regions between nucleosomes, resulting in fragments of approximately 180 to 200 bp (Kerr and Harmon, 1991). On agarose gel electrophoresis there is a characteristic ladder pattern. The endonuclease responsible for this biochemical correlate of apoptosis has not been well characterized. A candidate enzyme is an endonuclease that is activated by calcium in thymocytes (McConkey, et al., 1988). Increases in cellular calcium concentration can induce apoptosis (Kizaki, et al., 1989).

Gobé et al. (1990) made the kidney ischemic by partially occluding the renal artery. They found that during the first 2 to 8 d after partially occluding the renal artery, both necrosis and apoptosis of renal epithelial cells could be seen. From 10 to 28 d after clip placement, during the time when there was marked reduction in renal mass, there was continued cell death, but only apoptosis was observed. Schumer and colleagues (1992) examined kidney tissue at various times after 5, 30, or 45 min of ischemia. A small number of apoptotic bodies were found 24 and 48 h after reperfusion following 5 min of ischemia with no associated necrosis. In the kidney whose renal artery had been clamped for 30 or 45 min, apoptosis was found at 12 h after reflow, and was more prevalent after 24 and 48 h of reperfusion. A ladder pattern of DNA fragmentation was observed 24 h after 30 min of ischemia. As the period of ischemia was lengthened there was increased evidence for necrosis.

With ischemia and reperfusion there is the induction of a gene encoding sulfated glycoprotein-2 (SGP-2) (Rosenberg and Paller, 1991), a gene which has been implicated in apoptosis in the prostate (Buttyan, 1991). The control mechanisms responsible for the decision of a cell to die via an apoptotic process, or proliferate to replace adjacent cells that have died, will hopefully become better understood as cellular signaling and genetic mechanisms for "programmed cell death" are better defined.

Apoptosis has been seen in clinical acute renal failure. It seems to be particularly prevalent in posttransplant acute tubular necrosis, where it coexists with necrosis (Olsen, et al., 1989). Whether or not this reflects fundamental differences in the pathophysiology of ischemic acute renal failure between a native kidney and a transplanted organ is not clear at the present time (Olsen, et al., 1989). Joel Weinberg and one of the authors (JVB) have recently reviewed the pathophysiological factors implicated in ischemic acute renal failure in the transplanted kidney (Bonventre and Weinberg, 1992).

MOLECULAR ASPECTS OF HYPOXIA AND ISCHEMIA

We have discussed some of the factors which are important for cell injury with ischemia. In Figure 3 we present a general scheme of the injury and repair process in the kidney. There are likely to be a number of cellular and molecular responses to the ischemic insult which the kidney brings into play to alleviate or prevent the cell damage. In addition, the response of the kidney to ischemic injury is particularly interesting, since this organ has the ability to completely recover from an insult which results in a large degree of cell death and organ dysfunction. Surviving cells can proliferate and replace those that were irreversibly damaged. Whether this potential for proliferation exists in all surviving cells or only a subpopulation of "stem" cells is not certain at this time.

GENETIC RESPONSE TO OXYGEN DEPRIVATION

Oxygen deprivation is a widely encountered phenomenon in biology. Lower organisms like prokaryotes are very well adapted to this kind of "stress" and have developed special "defense" mechanisms to deal with it. Eukaryotes also have developed mechanisms to deal with low oxygen pressure. Phylogenetically highly developed organisms such as vertebrates are more vulnerable to hypoxia, although some have evolved mechanisms to deal with long periods of oxygen deprivation. Turtles and whales, for example, can dive for 30 min or longer (Hochachka, 1986).

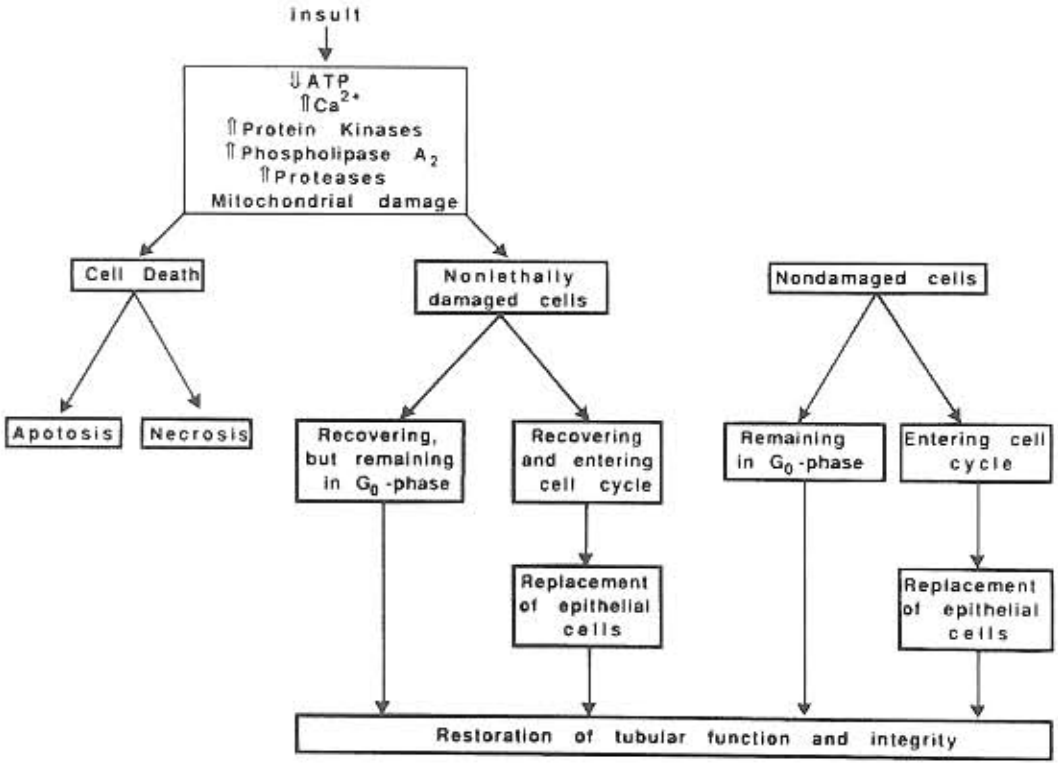

Figure 3 Schematic representation of ischemic injury and repair. Some of the factors likely involved in the initial injury to the tissue are listed in the top box. Protein kinases are listed here because they may be involved in covalent modification of proteins such as PLA_2, whose activity would then be increased. Cells can die an apoptotic or necrotic death. Nonlethally damaged cells can recover and remain in the Go stage of the cell cycle or may enter the cell cycle and replace dead cells. Cells not affected in a major way by the ischemia may likewise either remain dormant or enter the cell cycle to replace epithelial cells lost as a result of the ischemia. What determines whether a cell will remain in Go or will enter the cell cycle is undetermined and of great interest. Some of the possible mechanisms for the control of this are discussed in the text.

Some bacteria are well suited to a hypoxic environment, and it is likely that some of the genetic features characterizing their response to hypoxia will have homologs in higher organisms. *Escherichia coli*, as a facultative anaerobic bacterium, can use O_2 as electron acceptor, but can also use other substrates such as fumarate or nitrate (see Haddock and Jones, 1977, for a review). FNR, the product of the *fnr*-gene, is essential for the bacteria to grow under anaerobic conditions (Shaw and Guest, 1982a). The gene has been cloned and sequenced and was shown to exhibit homologies with other *E. coli* transcriptional regulator proteins (Shaw and Guest, 1982b). Organisms overexpressing this gene overproduce fumarate reductase. Thus the gene product functions as a positive regulator for expression of anaerobic energy-generating systems.

Another mechanism by which an organism can protect itself against hypoxia and reoxygenation is to increase its defense against ROS. Catalase and superoxide dismutase are induced in *E. coli* during shifts from anaerobic to aerobic environments (Hassan and Fridovich, 1977a, 1977b). Treatment of wild-type *Salmonella typhimurium* with 60 μM H_2O_2 for 60 min makes the bacteria resistant to subsequent treatment with 10 mM H_2O_2 or heat (Christman, et al., 1985). This protection can be blocked by chloramphenicol, indicating that protein synthesis is necessary. The synthesis of 30 proteins was increased as a result of the treatment with H_2O_2. One mutant (*oxyR1*), was highly resistant to killing by H_2O_2, heat, and organic hydroperoxides. *oxyR1* constitutively overexpressed 9 of 12 proteins normally induced soon after H_2O_2 exposure in the wild-type bacteria. These nine proteins included catalase (45 to 50 times higher activity), peroxidase, superoxide dismutase, glutathione reductase, NAD(P)H-dependent hydroperoxide reductase, and three heat-shock proteins. The *E. coli* gene encoding the OxyR protein was subsequently cloned (Tao, et al., 1989). In its NH_2-terminal region the protein shows a high

homology to other regulatory proteins in *E. coli* probably representing a helix-turn-helix motif of DNA-binding proteins. A closer look at the mechanism by which OxyR turns on the oxidative stress-defense genes revealed that the level of OxyR was not altered after treatment with H_2O_2 (Storz, et al., 1990). Also, strains that overexpress OxyR do not show increased expression of defense genes. These findings suggested a posttranslational modification of OxyR. Indeed, when OxyR protein was prepared from bacteria without DTT or with 1 m*M* DTT, OxyR was able to turn on transcription *in vitro*, whereas it was no longer able to do so if isolated in the presence of 100 m*M* DTT. When the DTT was removed by gel filtration, the OxyR protein was again active (Storz, et al., 1990). The increased transcriptional activity of the oxidized form of OxyR is probably not due to a higher DNA-binding affinity, but to a conformational change of the already bound protein, as shown by a different footprint pattern in the presence of 1 m*M* vs. 100 m*M* DTT. A model was proposed in which H_2O_2 diffuses into the cell, oxidizes OxyR, and thereby activates it (Storz, et al., 1990).

In mammals, a number of genes have been found to be induced by hypoxia or ischemia. *Histone H1°* mRNA accumulates transiently (3 h into anoxia, peaks at 6 h, decreases at 12 h) in Ehrlich ascites tumor cells exposed to 95% argon/5% CO_2 (Bouterfa, et al., 1990). By differential screening of a shock liver cDNA library from swine (4 h of cardiogenic shock and 4 h of recovery), higher levels of *metallothionein* mRNA were found (Buchman, et al., 1989). Another interesting set of experiments was performed to examine the mRNA levels of glycolytic enzymes in rat skeletal muscle. Rat skeletal muscle myoblasts grown under 93% N_2/2% O_2/5% CO_2 show a higher overall transcription rate under anoxic conditions. In particular, the rate for *triosephosphate isomerase*, *aldolase*, *pyruvate kinase*, and *lactate dehydrogenase* increased by day 1 and reached a steady state by day 3, while that for *glyceraldehyde-3-phosphate dehydrogenase* and *cytochrome c dehydrogenase* decreased (Webster, 1987).

The *erythropoietin* gene also is turned on by hypoxia. This system has been studied in more detail and therefore serves as an important example of a mammalian gene regulated by hypoxia. Erythropoietin (Epo) promotes growth and differentiation only in the red cell lineage. Though it was known for a long time that Epo is produced in the kidney, only very recently was the location of the Epo-producing cell in the kidney demonstrated (Koury, et al., 1988; Lacombe, et al., 1988). Koury et al. reported that the more severe the anemia in an animal the higher was the number of Epo-producing cells and not, as one might expect, the level of mRNA per cell (Koury, et al., 1989).

The investigation of the regulation of Epo production was greatly aided by the establishment of an *in vitro* model by Goldberg, Bunn and co-workers (1987). Two human hepatoma cell lines, Hep3B and HepG2, constitutively express Epo, and under the influence of hypoxia or cobalt can be stimulated to synthesize more of this cytokine (Goldberg, et al., 1987). Schuster et al. exposed rats to subcutaneous injections of cobalt or to anemia/hypoxia by lowering their hematocrits to 18 to 24% and by placing them into a hypobaric chamber (0.4 ATM) (Schuster, et al., 1989). In either case within 4 h there was evidence for a significant increase in transcription of the *Epo* gene in nuclear run-on transcription assays. Similar experiments were performed with the Hep3B cell line (Costa-Giomi, et al., 1990). Nuclear extracts prepared from Hep3B cells grown at 1% O_2 had higher levels of *Epo* transcription than seen in nuclei prepared from Hep3B cells grown at 21% O_2 or from HeLa cells (in the latter case it did not matter whether they were grown under hypoxic conditions or not).

The nature of this oxygen sensor is still not clear, although there is some indirect information regarding the sensor. Besides hypoxia and cobalt, nickel and manganese also stimulate transcription from the *Epo* gene. For both the induction by hypoxia and cobalt ions, protein synthesis was necessary (Goldberg, et al., 1988). The authors suggested that a rapidly turning over heme protein is key in the transcriptional regulation since nickel, cobalt, and manganese can substitute for iron in the porphyrin ring. These ions, in contrast to iron, cannot bind O_2, so that the heme protein would be locked in its deoxy state and subsequently turn on transcription. New protein synthesis is required because these ions cannot be incorporated into an already existing heme protein. The hypothesis of a heme protein as a common pathway through which all these agents act is supported by the observations that (i) there is no additive effect for hypoxia plus cobalt, hypoxia plus nickel, or cobalt plus nickel; (ii) carbon monoxide, to a great extent, abolishes Epo expression by hypoxia (carbon monoxide binds tightly to reduced heme protein and locks it in the oxy state); (iii) carbon monoxide does not abrogate the effects of nickel or cobalt (carbon monoxide binds only to reduced iron in the porphyrin ring); and (iv) inhibitors of heme synthesis also decrease the response of Hep3B cells to either hypoxia, nickel, or cobalt.

The situation is complicated, however, by the fact that post-transcriptional events also play a role. When Hep3B cells are grown at 1% O_2 and are transferred subsequently to 21% O_2, the steady-state

mRNA level of *Epo* declines by 50% between 90 and 120 min after reoxygenation (Goldberg, et al., 1991). This represents a maximal estimate of the actual half-life because new mRNA synthesis was not blocked. However, when mRNA synthesis was blocked by actinomycin D, instead of shortening the steady-state half-life of *Epo* mRNA the half-life was prolonged to 7 to 8 h (similar findings were made with cycloheximide). This suggested that a protein with a high turnover rate played a role in the degradation of *Epo* mRNA.

The cis-regulatory elements involved in hypoxic regulation of the gene appear to be situated both at the 5′- and 3′-ends of the gene (Goldberg, et al., 1988). When one compares the murine and human *Epo* genes, strong homology in the coding region is found. More surprising, however, is that the same high degree of homology was found in parts of the noncoding sequence: (i) 140 bp upstream of the transcription initiation site, (ii) in two regions in the first of four introns, and (iii) about 100 to 220 bp 3′ to the stop codon. Constructs, which utilized the conserved sequences located in the 5′-end of the gene, conferred hypoxia- as well as cobalt responsiveness to a vector with a growth hormone reporter gene. The addition of the conserved 3′ noncoding sequence increased the growth hormone level even further (Goldberg, et al., 1988).

HEAT SHOCK

Exposure of eukaryotic cells to a variety of stresses, including ischemia and reperfusion, thermal and oxidative stress, viruses, calcium ionophores, and heavy metals induces a remarkably conserved "heat-shock response" (Lindquist, 1986). This response is also seen with hypoxia and reoxygenation in *Drosophila* larvae. The "heat-shock response" includes the preferential synthesis of "heat-shock proteins" while generalized cellular protein synthesis is suppressed. We have reported (Polla, et al., 1991) that the mRNA levels of a member of the *HSP-70* gene family are increased soon after an ischemic insult to the kidney (Figure 4). mRNA levels are maximal at 3 h after clamping the renal artery for 40 min. Similar findings were subsequently reported by Van Why et al. (1992). Others had previously reported expression of the heat-shock response with ischemia-reperfusion in the liver (Cairo, et al., 1985) and heart (Currie, 1987). Van Why and colleagues localized the HSP-72 protein within the kidney postisch-

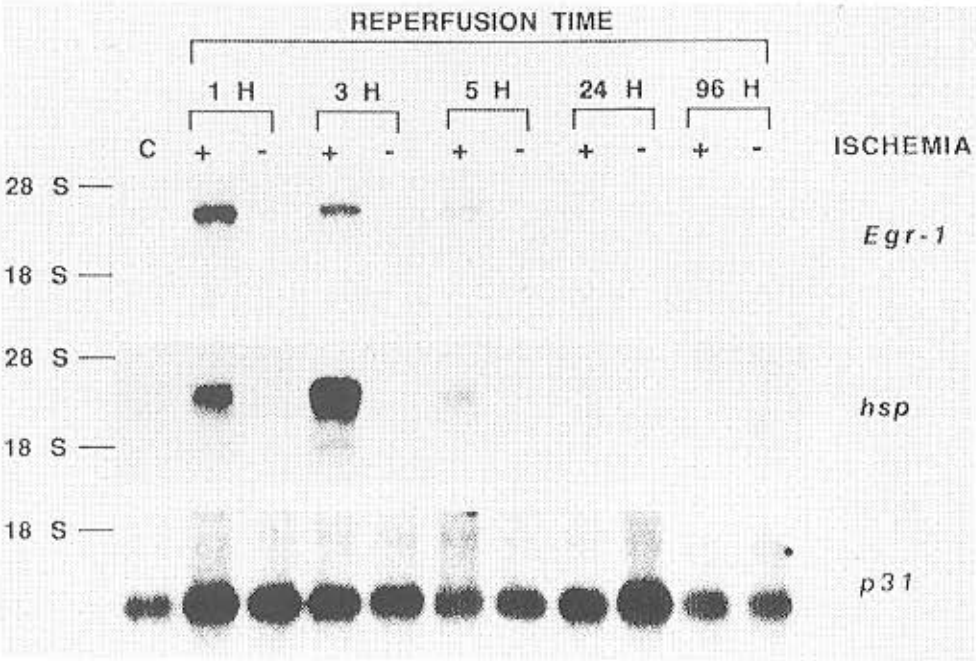

Figure 4 Northern blot analysis of *Egr-1* and *HSP-70* mRNA accumulation in rat kidney after 40 min of unilateral ischemia and varying periods of reperfusion. Total RNA was prepared from control kidneys (C), postischemic kidneys (+), and contralateral nonischemic (−) kidneys removed at varying times after reperfusion. Blots were hybridized with *Egr-1* or *HSP-70* cDNA probes. The blots were also hybridized with a cDNA probe encoding a "housekeeping" protein (P31). Positions of 18S and 28S rRNA are marked. These data have been reported in (Bonventre, et al., 1991) and (Polla, et al., 1991).

emia. HSP-72 is localized to the apical membrane of proximal tubule cells at 15 min of reoxygenation after 45 min of ischemia (Van Why, et al., 1992). The protein is distributed throughout the cytoplasm in a vesicular pattern from 2 to 6 h of reperfusion when microvilli begin to recover, and is located away from the apical membrane at 24 h of reperfusion. The early localization of HSP-72 to the apical part of the proximal tubular cell positions this protein at the site of the cell undergoing rapid changes during the early period of ischemia. As described previously there is rapid loss of apical microvilli with ischemia and loss of cell polarity.

Our laboratory has reported that the heat-shock response protected cultured neurons against amino acid excitotoxicity, which is believed to be the effector mechanism of ischemic injury to the brain *in vivo* (Rordorf, et al., 1991). Glutamate-induced excitotoxicity was inhibited by preheating the cells to 42.2°C for 20 min. This protection was afforded to cells subsequently exposed to glutamate 3 or 24 h after they were heated. Protection required new protein synthesis. It has been reported that the heat-shock response protects the pig kidney against warm ischemia (Perdrizet, et al., 1993). In this latter study donor kidneys were heated to 42.5°C *in vivo* for 15 min, allowed to recover for 4 to 6 h, and then exposed to 90 min of ischemia at 37 to 38°C. The kidneys were then removed, flushed with Euro-Collins solution at 4°C, and stored at this temperature for 20 h prior to transplantation into a littermate. Compared with controls, the heat-shock group had significantly greater survival rates and improved renal function on day 6 after the allograft was placed into the recipient. The cellular mechanisms responsible for protection after heat shock are not known, although the multiple cellular events that heat-shock proteins can influence suggest multiple possible mechanisms for this protection (Lindquist, 1986).

IMMEDIATE-EARLY GENES AND PROTO-ONCOGENES

There are a number of other genes which are induced with ischemia, for which it is not clear that the regulation is due to hypoxia itself or if the genes are induced by other factors present in the ischemic tissue. Primary human skin fibroblasts exposed to anoxia expressed C-Fos, but a number of other proto-oncogenes, including *c-abl, c-erbB, c-fes, c-fgr, c-mos, c-myc, c-Ha-ras, c-sis,* and *c-src,* were not induced (Deguchi, et al., 1987). This model likely does not reflect what happens *in vivo* with ischemia, since fibroblasts in culture are relatively resistant to oxygen deprivation. *c-fos* is also induced within 1 h after ischemia in the rat and mouse kidney (Ouellette, et al., 1990; Safirstein, et al., 1990b) and rat liver (Schiaffonati, et al., 1990). *c-fos* induction is transient with mRNA levels decreasing by 4 h and clearly at baseline by 24 h of reperfusion.

In addition to *C*-Fos, another immediate-early gene, *Egr-1,* is also induced in the postischemic kidney (Ouellette, et al., 1990). Like C-Fos, the expression of Egr-1 is also transient, but mRNA levels are still slightly elevated at 24 h in the mouse and at control levels after 24 h in the rat (Bonventre, et al., 1991; Ouellette, et al., 1990; Safirstein, et al., 1990b). We have localized the Egr-1 protein to the nuclei of the thick ascending limb, whereas no protein was seen in the more severely affected S3 segment of the proximal tubule (Bonventre, et al., 1991) (Figure 5). The implications of the expression of immediate-early genes is still unclear. Since both *c-fos* and *Egr-1* encode transcription factors (Sukhatme, 1990; Verma and Sassone-Corsi, 1987) it suggests that the early activation of transcription factors and subsequent activation by them of genes located downstream in a cascade of genetic events is important for the kidney postischemia. Precisely what this cascade is important for is not clear. The Egr-1 protein is localized to a tubular segment which is not severely damaged in the rat model of ischemia. Immediate-early gene expression may reflect a cellular response to the dedifferentiating influences of ischemic injury (Molitoris, et al., 1988). This loss of polarity represents a significant stress to the cell. If the cell is damaged significantly it may not survive, and proliferation of adjacent cells may be necessary to restore epithelial integrity and function. If the damage is not severe enough to kill the cell, however, the cell may respond to the insult with a "differentiation" response. In this way the cell restores differentiated function that was altered due to the ischemia. For example, cell polarity is altered in the nonlethally damaged cell, and it is likely that a new sequence of genes is induced to restore this polarity as the cells recover. Indeed, the *Egr-1* gene is induced when differentiation is induced in heart and nerve cell cultures (Sukhatme, et al., 1988). *Egr-1* is also induced by epidermal growth factor (EGF) in cell culture (Sellmayer, et al., 1991), and Humes et al. (1989) have shown that EGF enhances renal tubule cell regeneration and repair. Perhaps the fact that the thick ascending limb can accumulate the Egr-1 protein represents an adaptive response with protective consequences for cell and nephron segment integrity.

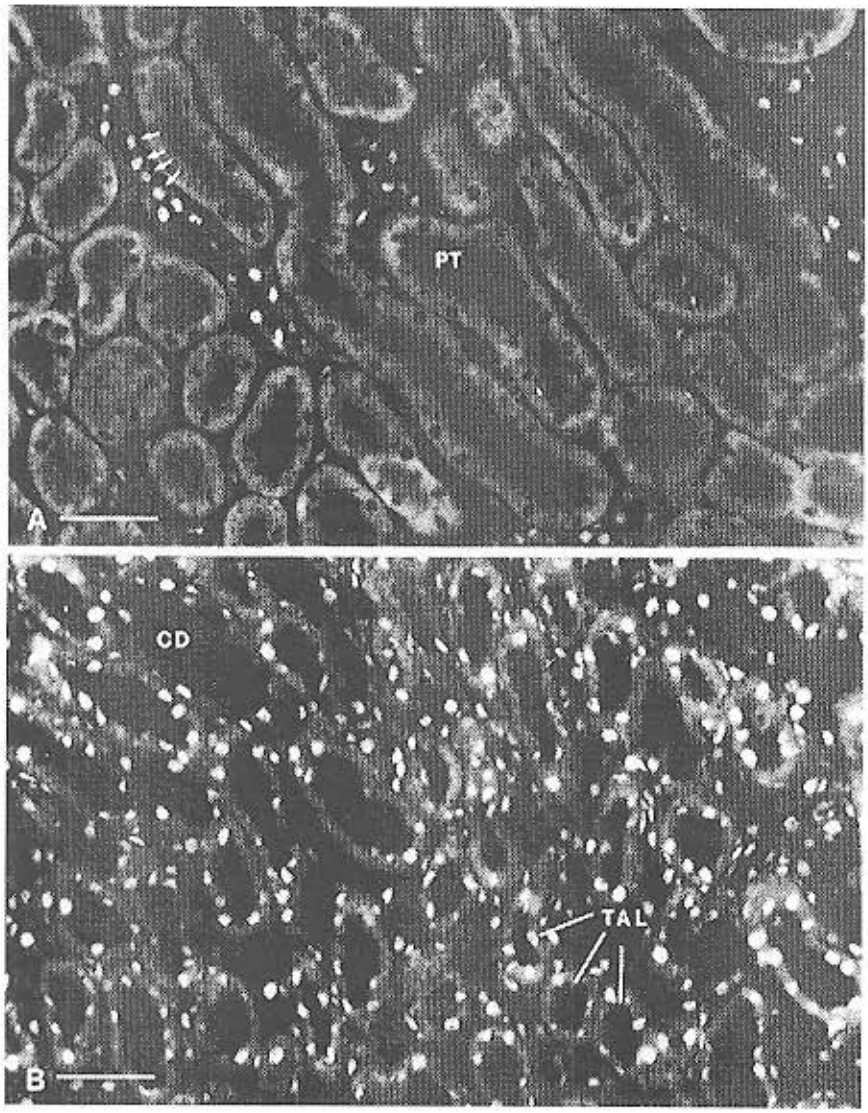

Figure 5 Cryostat sections of outer medulla of ischemic rat kidney after 1 h of reperfusion, demonstrating the localization of the Egr-1 protein. (**A**) In the outer stripe of outer medulla there is positive staining of nuclei in thick ascending limbs (arrows) and collecting ducts. The nuclei of straight proximal tubules in the outer stripe are not stained. (**B**) In the inner stripe of outer medulla there is heavy nuclear staining of thick ascending limbs (TAL) and collecting ducts (CD). In addition, the nuclei of some thin limbs and capillaries also demonstrate enhanced Egr-1 staining. Bar = 50 μm. (From Bonventre, et al. (1991) *Cell Regul.* 2:251–260. With permission.)

GROWTH FACTORS

An important component of the repair process in the kidney may involve the local induction of genes encoding growth factors which then act in a paracrine or autocrine manner to enhance epithelial cell regeneration. Toback (1992) has recently written an excellent review of the possible roles played by growth factors in the regenerative response after acute renal failure. If primary cultures of human umbilical vein endothelial cells are exposed for 3 d to 1 or 0% oxygen, the *platelet-derived growth factor-B (PDGF-B)* mRNA levels increase by eight- to tenfold (Kourembanas, et al., 1990). This increase in mRNA level is due to a higher transcription rate, though the half-life of the *PDGF-B* mRNA is also slightly prolonged during exposure to 1% oxygen (90 vs. 60 min in 21% oxygen). The cells survived under these conditions of low oxygen tension, although they had a somewhat longer doubling time. In

addition, reoxygenation was not necessary for significant changes in mRNA levels. The level of *PDGF-A* mRNA was not affected, so that it is likely that increased amounts of PDGF-BB homodimers are produced in these cells. While PDGF likely does not directly enhance the growth of kidney epithelial cells, this growth factor may induce fibroblasts to release factors which may act on the epithelial cell to enhance proliferation.

Another growth factor that may be important for the recovery of the kidney after an ischemic insult is EGF. EGF is a potent mitogen for kidney epithelial cells. With ischemia there is a marked reduction in levels of *preproEGF* mRNA along with reduced urinary excretion of EGF (Safirstein, et al., 1990a). However, binding of [^{125}I]EGF increased in both cortical and medullary tissue 24 h after reflow (Safirstein, et al., 1990a). Two groups have found that exogenous EGF enhances recovery of the kidney postischemia and this enhanced recovery is associated with enhanced thymidine uptake into the kidney (Humes, et al., 1989; Norman, et al., 1990). Another group, however, has failed to demonstrate effects of exogenous EGF on recovery from ischemia as measured by either renal function or mitotic indices (McKanna, et al., 1992).

Transforming growth factor-alpha (TGF-alpha) is a 50-amino acid peptide which exerts its biological effects via the EGF receptor (Massagué, 1990). Developing mesonephric tubules, renal cell carcinomas, and other human tumors produce TGF-alpha (Wilcox and Derynck, 1988). TGF-alpha may derive from infiltrating macrophages in the postischemic tissue (Rappolee, et al., 1988), and then act upon epithelial cells to promote mitogenesis.

There is enhanced renal synthesis of insulin-like growth factor I (IGF-1) after renal ischemia (Anderson and Jennische, 1988; Matejka and Jennische, 1992). Miller et al. have reported that administration of IGF-1 accelerates recovery of rat serum creatinine levels after an ischemic insult (Miller, et al., 1992). Since we believe that recovery from acute renal failure may recapitulate many aspects of renal development, it is interesting that IGF-1 has been proposed to play a role in metanephros development (Rogers, et al., 1991).

Kid-1

We have recently identified a new transcription factor (Figure 6), which is primarily expressed in the kidney and whose mRNA decreases in content in the postischemic kidney (Figure 7) and kidney after folic acid administration (Witzgall, et al., 1993). Folic acid induces acute renal failure with marked

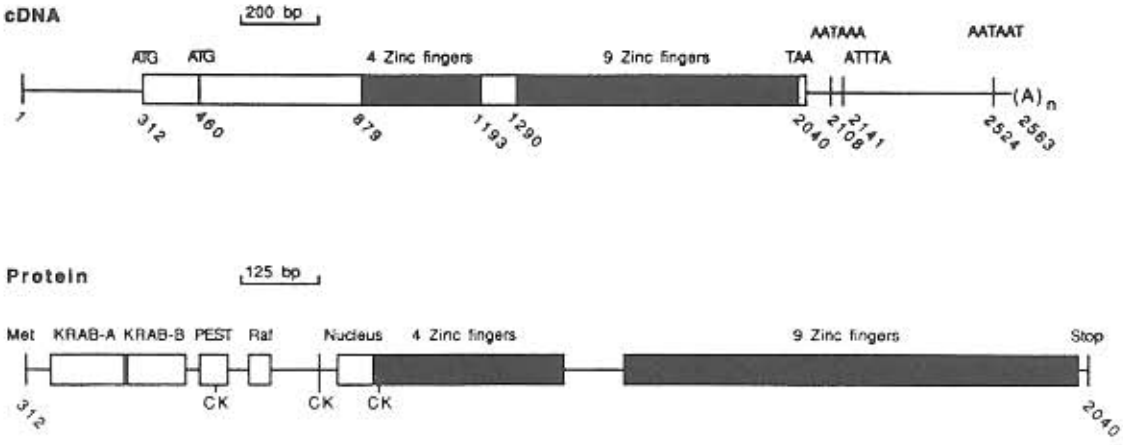

Figure 6 Structural characteristics of *Kid-1* cDNA and predicted protein product. The coding sequence is shown from the putative start codon at position 312 bp. There is a possible alternative start site at position 460. There are 13 zinc fingers divided into two groups of 4 and 9. Putative polyadenylation sequences (AATAAA, AATAAT) and an instability sequence (ATTTA) can be found in the 3'-untranslated region of the cDNA. The predicted protein contains a Krüppel associated box-A and -B ("KRAB-A, -B"), which are regions conserved in approximately one third of all zinc finger proteins and whose function is unknown. The protein also contains a PEST (proline, glutamic acid, serine, and threonine-rich) region, which is present in many proteins with short half-lives. In addition there is a raf-homology domain (Raf), a region highly homologous to a 12-amino acid region in all members of the Raf family of protein kinases. There is a nuclear translocation signal ("Nucleus"), and casein-kinase II-consensus sites (CK). (Taken from Witzgall, et al. (1993) *Mol. Cell. Biol.* 13: 1933–1942. With permission.)

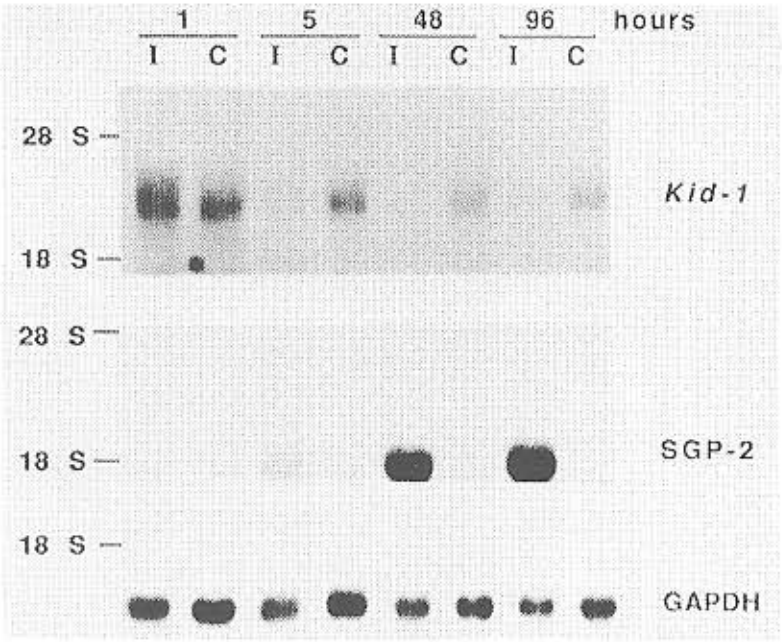

Figure 7 Northern analysis of kidney RNA after ischemia and varying periods of reperfusion. Total RNA was taken from kidneys exposed to 30 min of ischemia and various times of reperfusion or from the contralateral control kidneys. RNA was hybridized with either *Kid-1*, SGP-2, or GAPDH cDNA probes. At each time point "I" represents RNA taken from an ischemic kidney and "C" RNA taken from the contralateral control kidney. (Taken from Witzgall, et al. (1993) *Mol. Cell. Biol.* 13: 1933–1942. With permission.)

tubular cell injury. This cDNA encodes a predicted protein with 13 zinc fingers, which implies that it binds to DNA and may regulate transcription. We have called this new gene *Kid-1* because it is found primarily in the *k*idney, is modulated in expression by *i*schemia, and is *d*evelopmentally regulated. Its expression increases with increasing time of postnatal development (Figure 8). Therefore, *Kid-1* expression is decreased when kidney proliferation occurs during the regeneration phase after acute tubular injury, and early in postnatal development when the kidney of the rat is undergoing rapid proliferation (Saxen, 1987). The structure of the Kid-1 protein suggests that it might be a substrate for phosphorylation. In addition, there is a 12-amino acid region that is highly homologous to all members of the Raf-1 family of protein kinases. When a chimeric construct including the nonzinc finger region of Kid-1 and the DNA-binding region of yeast GAL4 is transfected into kidney cells along with chloramphenicol acetyl transferase reporter plasmids containing GAL4 binding sites, there is marked suppression of

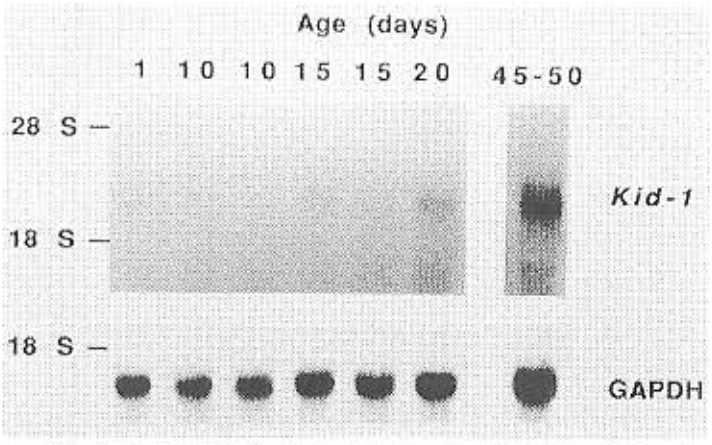

Figure 8 Northern analysis of total RNA collected from rats at varying stages of postnatal development. This blot was hybridized with *Kid-1* and GAPDH cDNA probes. Total RNA was taken at various postnatal ages. Note that Kid-1 is expressed at the highest level in the oldest animals and the level of expression increases with increasing age. Hybridization with GAPDH shows approximately equivalent levels of total RNA in each lane. (Taken from Witzgall, et al. (1993) *Mol. Cell. Biol.* 13: 1933–1942. With permission.)

transcriptional activity, suggesting that the Kid-1 protein is a transcriptional repressor. It is possible that genes such as *Kid-1* are involved in the complex integration of genetic signals necessary to induce dedifferentiation, proliferation, and then differentiation that is required for the renal tubular epithelium to restore its anatomical and functional integrity after an ischemic insult.

CONCLUSIONS

As reflected in the above discussion there are various factors influencing cell death and repair. It is likely that many of the factors we have discussed do not work in isolation. They interact in complicated, poorly understood ways and these interactions, together with the nonspecificity of interventions 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. Hopefully it will be the case that one or two of these factors will be most important, and even when synergy occurs, one or two factors will be primary. Hence therapeutic intervention directed at these one or two factors will be successful in preventing cell death. Since prevention of cell death will not always be possible, since the insult cannot be predicted in man, it is very possible that it will be just as important to hasten recovery of the damaged tissue in order to make important advances in patient survival. We believe that recovery from ischemic acute renal failure is likely regulated by a number of genetic events, some of which involve the upregulation of genes important for dedifferentiation and proliferation, and others involving the downregulation of genes which are important for differentiation and growth arrest. Some of the genes involved may be kidney specific and others not.

In summary, the pathophysiology of ischemic acute renal failure is complex. The unacceptably high morbidity and mortality dictate that we continue a diligent search for a better understanding of the mechanisms responsible for ischemic cell death. Such knowledge may be applicable to treatment of other organs frequently affected by ischemia—the heart and brain. At the same time we must continue to explore basic mechanisms responsible for renal repair, since the kidney has the potential to completely recover and by hastening this process we hopefully will be able to enhance patient survival.

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