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Role of calcium and related mechanisms in ischemic tubular epithelial injury

T. J. Burke, Ph.D.; A. Kribben, M.D.; J. F. M. Wetzels, M.D.; L. Yu, M.D.; E. D. Wieder, Ph.D.; and R. W. Schrier, M.D.
Department of Medicine. University of Colorado School of Medicine. Denver, Colorado.

Background

In 1981, at the VIIIth meeting of the International Society of Nephrology, a hypothesis concerning the role of calcium (Ca^{2+}) ions in mediating the functional, biochemical and morphologic injury which characterize ischemic acute renal failure (ARF) was presented[1]. In the succeding 10 years, studies by many laboratories have provided a stronger, more complete understanding of the particular processes by which Ca^{2+} ions are involved in cellular injury. This review will synthesize these observations and provide a comprehensive view of our current impressions as to how, when and where Ca^{2+} is involved in the cellular damage that, during and following ischemia, ultimately results in functional renal injury.

Oxygen deprivation

In vivo oxygen deprivation occurs in several clinical circumstances and these can be mimicked in experimental models. The most severe form of oxygen deprivation, that is complete ischemia, occurs clinically when vessels are totally occluded during, for example, surgical procedures involving aortic clamping. Blood flow and thus oxygen and nutrient delivery to organs or tissues downstream from the occlusion is abruptly interrupted and accumulated metabolic toxins are not carried away. Experimentally, these situations can be modelled by placing totally occlusive, mechanical clamps on selected vessels in intact animals.

This experimental model therefore provides a precise correlate of an *in vivo* ischemic event that does occur clinically. However, total, complete cessation of renal blood flow occurs in only a small number of those clinical states that result in «ischemic» acute renal failure. More often, very severe (but not complete) reductions in renal blood flow occur due, in part, to either humorally or toxin mediated vasoconstriction. These perturbations lead to reduced delivery of oxygen to organs, including the kidney. In addition, ventilation defects and the inability of pulmonary tissue and vessels to permit ful oxygenation of erythrocytes can reduce tissue oxygen delivery even when blood flow to organs is relatively normal. Both of these conditions result in decreased oxygen delivery — and are designated as hypoxia. However, since the oxygen extraction by the kidney is quite low, clinically relevant renal hypoxia occurs primarily secondary to decreased perfusion. Necessarily then, hypoxia is a rather imprecise term reflecting a range of oxygen levels somewhere between no oxygen at all (anoxia) and normal oxygen levels.

Based on an evaluation of the literature over the last 10 years, some of the controversy concerning mechanisms of injury and modes of protection may relate to differences in the degree of oxygen deprivation that were achieved in particular studies as well as differences in models and species. In the following sections, we will identify the levels of oxygen deprivation (anoxia or hypoxia), models and species in a particular study so as to permit a more complete interpretation of the reported results.

Calcium channel blockers

Our initial report of protective effects of calcium channel blockers (CCB) against norepinephrine induced ischemic ARF in the dog[2] was followed shortly thereafter by a more complete description of these results[3]. Corroborative results were also obtained by investigators who place mechanical clamps on the renal arteries of both rats and dogs[4, 5]. Following these early, encouraging results —others have also shown protection in these or similar models— including organ transplantation studies in both animals and humans[6, 7]. In fact, very few negative results regarding the protective effects of CCB have been reported and these are usually characterized (1) by the use of doses of NE that are used in dogs rather than rats[8], (2) by the use of anesthetic agents such as inactin which are seldom used in dogs and have vascular effects that may obscure protection by CCB[8, 9], (3) by prolonged renal artery clamping (<45-50 min) and/or (4) by very short (1-2 minu-

Correspondencia. Robert W. Schrier, M.D.
Professor and Chairman, Department of Medicine; C281, University of Colorado School of Medicine.
4200 East 9th Avenue.
Denver, CO. 80262. U.S.A.

tes) exposures of the renal circulation to the CCB[9]. It is also clear that intrarenal infusion of CCB allows for higher renal concentrations while avoiding systemic vasodilatation.

Rats anesthetized with the long-acting anesthetic inactin and then infused with verapamil for 2 minutes prior to placing clamps on the renal arteries, exhibited no protection at 24-48 hours of reflow[9]. Goldfarb et al. had previously shown that a 15-minute infusion of verapamil in rats anesthetized with pentobarbital and clamped for 70 minutes did not improve renal function after 24 hours of reflow[4]. As noted earlier, however, we have reported that verapamil is protective in pentobarbital anesthetized dogs if this CCB is infused for 20-30 minutes prior to NE infusion into the renal artery or for 2 hours (but not 1 hour) following cessation of the NE infusion[3]. Also of interest, if Ca^{2+} is removed for 2 hours from the bathing media in which primary cultures of rabbit tubules are incubated following *in vitro* anoxia, cell viability is also enhanced[10]. Thus the timing, route and duration of the CCB infusion is important to the demonstration of protection. Clearly the results of any study must be interpreted and analyzed with perspective and care, be they *in vivo* or isolated *in vitro* studies. Only in this manner will true insight into the role of Ca^{2+} ions in the pathogenesis of oxygen deprivation be fortchcoming.

Renal tubules, vascular smooth muscle cells and endothelial cells exhibit damage during oxygen deprivation and this damage often increases during reflow. Our studies have therefore focused on the mechanisms by which altered Ca^{2+} homeostasis may contribute to the development of this injury. The difficulties associated with, and the many varied models that are often used for, *in vivo* experiments have led some investigators to pursue the independent effects of oxygen deprivation alone on the cell injury in renal tubules studies in isolation. Such studies provide for very precise control of (a) the degree of oxygen deprivation, (b) the ambient pH and (c) the substrate and nutrient availability. Thus, isolated tubule studies are experimentally powerful tools for identifying the causes of cell injury *in vivo* during severe or total oxygen deprivation.

Calcium ions and cell injury

The protective effects of calcium channel blockers (CCB) in several experimental and clinical settings associated with oxygen deprivation injury suggested, but certainly did not prove, that Ca^{2+} ions are an important factor for inducing ischemic tubular epithelial cell injury. *In vivo* studies cannot, however, distinguish between vascular and epithelial effects of CCB. Thus, *in vitro* studies of isolated renal tubules have been used to study the role of calcium in ischemic renal epithelial damage. There are several important studies that deserve attention with respect to the role of calcium is ischemic epithelial cell injury. Three approaches have been used in such studies: (1) removal of Ca^{2+} from the extracellular bathing medium, (2) addition of CCB and (3) addition of Ca^{2+} ionophores — agents that accelerate Ca^{2+} entry into cells. It has become apparent from the results of these studies that the level of oxygen deprivation, be it total and complete (anoxia) or partial but severe (hypoxia), may determine the changes in cellular calcium handling that occur in renal tubules. In the following sections, we will evaluate cellular Ca^{2+} dynamics in these two settings, *in vitro* anoxia and *in vitro* hypoxia.

Anoxia vs. hypoxia

One important observation contrasting hypoxic and anoxic injury and the potential role of Ca^{2+} ions is that of Takano et al.[11]. These authors reported that in rabbit proximal tubules complete anoxia was not associated with any increase in total tissue Ca^{2+} whereas severe hypoxia was characterized by progressive, time dependent increases in tissue Ca^{2+}. What do these results signify? First, it could be that severe hypoxia is the only situation in which continuous Ca^{2+} influx is prolonged, sustained and not paralleled by proportionate rates of Ca^{2+} efflux (due to the reduced cellular ATP that accompanies oxygen deprivation). Under these conditions then either cytosolic free Ca^{2+} (Ca^{2+}_i) would reach quite high levels (parralleling the increased tissue Ca^{2+} levels) or, alternatively the Ca^{2+} might be found to be sequestered in intracellular organelles. In this latter condition, therefore, Ca^{2+}_i would not be expected to rise to very high levels. However, in either situation total tissue Ca^{2+} would be elevated in association with hypoxia.

Takano et al. have used ruthenium red to block mitochondrial Ca^{2+} uptake during hypoxia; their results showed that tissue Ca^{2+} overload did not occur[11]. Thus, it appears as if Ca^{2+} entry into renal tubules during hypoxia is elevated for a substantial period of time. The mitochondria sequester this additional cellular Ca^{2+} burden. Weinberg and Humes have reported similar results, demonstrating that total tissue Ca^{2+} rises from approximately 10-15 nmol/mg protein during normoxia to 20 nmol/mg protein after 15 minutes of hypoxia and to 30 nmol/mg protein after 30 minutes of hypoxia[12,13]. Thus independent laboratories have, therefore, demonstrated time dependent tissue Ca^{2+} accumulation during hypoxia. Ca^{2+} accumulation with hypoxia may be due to either the accelerated entry of Ca^{2+} or diminished Ca^{2+} efflux rate or both events. This question was partly resolved by our laboratory when we showed that during severe hypoxia, Ca^{2+} entry into renal tubules was increased by 50-100 % within one minute after pO_2 was reduced to low levels[14]. In that study, the high Ca^{2+} uptake rate remained elevated for at least 20 minutes. Therefore, although Ca^{2+} efflux rate is likely to be decreased because cellular ATP levels are reduced quite promptly during oxygen deprivation, Ca^{2+} uptake rate into hypoxic tubules is also increased. It is important to note that CCB prevent the early increase in Ca^{2+} up-

take rate and delay the development of cell injury in rat proximal tubules[15]. To summarize, *in vitro* hypoxia is accompanied promptly by increased rates of Ca^{2+} entry which are sustained for up to 30 minutes (or longer) and the mitochondria sequester this Ca^{2+} thereby resulting in progressive tissue Ca^{2+} overload.

It might be anticipated, based on these results, that Ca^{2+} would not increase more than a modest amount under hypoxic conditions and this expectation has recently been experimentally confirmed in a model of chemical hypoxia[16]. The absence of appreciable increases in Ca^{2+} during hypoxia is not unique to hypoxic renal tubules since it is observed in other tissues as well. For example, in cells of the carotid body, steady state hypoxia is accompanied by only very small increases in Ca^{2+} [17].

Together, these above results allow us to formulate the following hypothesis: During severe hypoxia, Ca^{2+} influx rate is increased above the normal level and, since it is likely that Ca^{2+} efflux rates are also simultaneously depressed due to the absence of sufficient ATP, the buffering of this additional cellular Ca^{2+} burden is provided by mitochondria (via a process sensitive to ruthenium red). The buffering by the mitochondria attenuates the potential rise in Ca^{2+}_i and leads simultaneously to mitochondrial Ca^{2+} accumulation. Preventing increased Ca^{2+} uptake with CCB suggests that at least a portion of the increased uptake occurs via a pathway or route similar to potential activated slow Ca^{2+} channels and moreover provides evidence that *in vivo* pretreatment with CCB may lessen renal injury via direct effects on renal tubular epithelium as well as by any effects to protect renal vascular integrity and/or renal hemodynamics.

Anoxic conditions, however, apparently provide a different series of stresses than do hypoxic conditions on the mechanisms responsible for cellular Ca^{2+} homeostasis. Specifically, the absence of mitochondrial participation in Ca^{2+} buffering distinguishes anoxia from hypoxia. Again, a historical perspective provides a foundation for interpreting the results of more recent studies that have confirmed and refined the hypothesis that anoxic injury has basis which is distinct in several aspects from hypoxic injury. For example, it is important to recognize that in addition to differences in ambient pO_2 between hypoxia and anoxia, Ca^{2+} uptake rate and mitochondrial buffering are also quite different. Specifically, during anoxia (pO_2 = 0 mmHg) there is no detectable increase in total tissue Ca^{2+} [13]. In densely packed renal tubules, wherein diffusion of pO_2 from the hypoxic medium to the tubules is unikely to occur, tissue Ca^{2+} overload also fails to occur even though the period of total *in vitro* oxygen deprivation is as long as 60 minutes[12]. In this latter model, extracellular pH of the medium surrounding the packed tubules decreases and this decrease in pH (acidosis) is suggested to be responsible for the absence of an increase in tissue Ca^{2+} [11]. Finally, even when extracellular pH is normal, stirred suspensions of anoxic tubules also fail to show increased levels of tissue Ca^{2+} [11]. Thus intracellular acidosis rather than extracellular acidosis may be the more important determinant of reduced tissue Ca^{2+} overload during anoxia[12]. These results, confirmed independently by different laboratories[11-13], correlate well with the results of *in vivo* studies. It is known, for example, that during norepinephrine-induced total renal ischemia or during renal artery clamping studies *in vivo*, there is no increase in total tissue Ca^{2+} in the kidney at the end of the ischemic period, that is, prior to reperfusion[3, 18]. Taken together, these *in vivo* and *in vitro* observations lead naturally to the question of whether or not there is an increase in Ca^{2+} influx in anoxia as there is in hypoxia. Our recent observations in isolated renal tubules[15] as well as those performed in cultured kidney cells[19], demonstrate increases in Ca^{2+} uptake that are similar in magnitude to those observed in severe hypoxia and are sensitive to CCB. However, and importantly, these increases in Ca^{2+} influx are not sustained as they are in hypoxia – rather Ca^{2+} uptake rates return to normal after 10 minutes of total oxygen deprivation[15]. Therefore, the absence of tissue Ca^{2+} overload during anoxia can be attributed, at least in part, to an abrupt termination of the increased Ca^{2+} uptake rate. We hypothesize that the reason for this return to normal rate of Ca^{2+} influx may be related to the development of intracellular acidosis, a condition that may develop more completely during ischemia or anoxia than during hypoxia as anoxic cells attempt to generate ATP from glycolysis[12].

If Ca^{2+} uptake rate is increased, even for a short time during anoxia, the next question is does this additional cellular Ca^{2+} burden become sequestered in the mitochondria? Based on the results of recent studies, this does not appear to be the case. Two separate observations complement those which show the absence of mitochondrial Ca^{2+} accumulation during anoxia. First, steady state anoxia is often accompanied by higher levels of cytosolic Ca^{2+} than occurs in hypoxia[17, 19]. The values, observed using fluorescent dyes are presumably not higher than they are, because Ca^{2+} uptake may be promptly arrested presumably, in part, by the development of intracellular acidosis. Such studies are difficult, however, to perform because the fluorescent dyes can leak from the oxygen deprived cells[20]. Thus the Ca^{2+} that does enter the anoxic cells albeit for only a short time, may remain sequestered in the cytosol. Second, Takano et al. had suggested that during anoxia the absence of a mitochondrial membrane potential would preclude Ca^{2+} sequestration in this organelle even if Ca^{2+} uptake was increased[11]. This reasoning is compatible with the absence of tissue Ca^{2+} overload during *in vitro* anoxia or *in vivo* total ischemia and would also explain the higher cytosolic-free Ca^{2+} during anoxia than hypoxia[17]. As noted above, the probable development of intracellular acidosis prevents Ca^{2+} uptake from remaining high during anoxia and thus contributes to only a rather small increase in Ca^{2+}_i and, importantly, no increase in total tissue Ca^{2+}. Imposition of acidotic condition during *in vitro* hypoxia also afford protection during reperfusion[21]. Specifically, we have shown that Ca^{2+} up-

take rate is lower during 60 min of reperfusion as are morphologic injury and LDH release, if hypoxia is induced for 30 min in a bathing medium of pH 6.9[21]. It is likely that the reduced Ca^{2+} uptake during hypoxia at pH 6.9 delayed what otherwise would have been tissue Ca^{2+} overload and phospholipase activation may also been delayed[21]. pH effects on phospholipases will be discussed below. Intracellular pH, however, has not yet been measured in both hypoxia and anoxia.

To summarize, anoxic injury to renal tubules *in vitro* appears to be a very appropriate model for *in vivo* total ischemia since in neither situation does tissue Ca^{2+} overload occur. *In vitro* anoxic studies do permit investigators to identify (a) a short lived increase in Ca^{2+} uptake that is apparently terminated at least in part by the development of intracellular acidosis, (b) a more pronounced increase in cytosolic free Ca^{2+} than is seen during hypoxia and (c) no detectable increase in mitochondrial or total tissue Ca^{2+} overload. The next question that arises from these observations is whether the Ca^{2+}, which does in fact enter anoxic cells only to be sequestered in the cytosol rather than in the mitochondria, contributes to the development of cell injury.

Ca^{2+} and cell injury

Information regarding extracellular Ca^{2+} and its role in anoxic cell injury has been obtained by Takano et al. These authors showed that a reduction in extracellular Ca^{2+} to 2.5 μM, —a 40-fold decrease compared to normal— reduced injury as assessed by a 50 % decrease in the release of lactate dehydrogenase (LDH)[11]. Thus, either removal of Ca^{2+} from the outside environment of anoxic cells[11] or addition of CCB, as we have recently shown[15], reduces anoxic cellular injury; these observations provide the strongest evidence to date that Ca^{2+} ions moving intracellulary during anoxia do initiate cell injury during oxygen deprivation and that cytosolic Ca^{2+} overload is not simply a secondary consequence of cell death.

Role of cytosolic Ca^{2+} in cell injury

The next important consideration with anoxia concerns how the apparently modest elevations in steady state Ca^{2+} result in cell injury and whether it is this concentration of Ca^{2+} or the continued high Ca^{2+} influx rate in hypoxia, that is more important in initiating cell injury.

Some investigators have attemped to define the role of small increases in Ca^{2+} in the process of cell injury. The results of such studies also add support to the hypothesis that Ca^{2+} may be a causal factor in the initiation of oxygen deprivation-induced renal cell injury. For example, Nakamura et al. have assayed the phospholipase A_2 (PLA_2) activity and activation at the end of a period of total renal ischemia in the rat[21]. Activity of PLA_2 was increased in the membrane fraction and decreased in the cytosolic pool. These authors also determined that the K_D for activation is a [Ca^{2+}] of about 500 nM with activation begining at 100 nM[22]. These concentrations, of course, would be within the ranges expected for Ca^{2+} during *in vitro* anoxic studies of suspensions of proximal tubules. (See above.)

Portilla and Mandel have shown that PLA_2 activity in rabbit renal tubules is: (a) increased during *in vitro* anoxia, (b) moves from cytosolic to membrane sites and (c) is accompanied by an increased mRNA signal for PLA_2[23]. Thus, in renal anoxia in both rat and rabbit renal cortical tissue, PLA_2 activity is increased prior to the initiation of reoxygenation or reflow. These data coupled with the profound decrease in LDH release when anoxic rabbit tubules are incubated in a low Ca^{2+} medium (2.5 μM), strongly suggest, that influx of Ca^{2+} ions from the extracellular fluid may result in activation of phospholipases whose activity then results in membrane damage as reflected by LDH release.

It is incumbent not only to show that PLA_2 activity is increased but that such activity is temporally accompanied by the expected alterations in phospholipids and/or fatty acids. To this end, Humes et al. have shown that fatty acids are detected in the medium and in the cells of hypoxic proximal tubules within 15 minutes of inducing hypoxia, i.e. well before any appreciable or significant change in the tubule phospholipid content[24]. We have confirmed this early increased release of fatty acids in hypoxic proximal tubules of the rat and relative stability of lysophospholipids[25]. Thus, *in vitro* hypoxia is associated with very significant increases in the release of fatty acids of a type, i.e. arachidonic acid, that are located at the S-n2 position of phospholipids and are susceptible to PLA_2 activity.

Three other observations confirm the importance of an early activation of phospholipases by Ca^{2+} ions as potential contributor to cell injury. First, Molitoris et al. have shown that *in vivo* ischemia induced in the rat by renal artery clamping for 50 minutes results in a slow decrease in phosphatidylcholine and phosphatidylethanolamine[26]. These data confirm those from Hume's group mentioned above which showed that while fatty acids accumulate promptly in hypoxic tubules, there is a much slower degradation of phospholipids. The subtle differences in the timing and magnitude of phospholipid changes may reflect methologic events, differences phospholipase activation during *in vivo* ischemia (anoxia) as compared to *in vitro* hypoxia, to a species difference or to a combination of these possibilities. In any case, taken together these data indicate that phospholipases are activated promptly during oxygen deprivation and that fatty acid release precedes measurable changes in individual or total phospholipid pools. In addition to these observations, Malis and Bonventre have demonstrate that mitochondrial phospholipases are probably activated in the presence of Ca^{2+} in a model of oxidant injury as could occur during hypoxia itself and with reoxygenation[27]. They demonstrated an attenuation of injury to mitochondria when dibucaine, a

phospholipase inhibitor, was present thus suggesting that phospholipases were activated in the presence of Ca^{2+}. Finally we have used the phospholipase inhibitors, mepacrine and dibucaine, and demonstrated a reduction in cell injury during hypoxia in rat proximal tubules[14].

In summary, the accelerated rate of Ca^{2+} influx into hypoxic or anoxic proximal tubules appears to provide for activation of phospholipases. It is likely that the intracellular concentration of Ca^{2+} of anoxic tubules may be somewhat higher than that in hypoxic tubules[28]; however, during anoxia damage is not higher than during hypoxia[11, 15]. This result may be partly due to an induction of intracellular acidosis which is either more prompt or more substantial than that which occurs in hypoxic tubules. PLA_2 activity is pH dependent, with an optimum pH value for activation of 8.5-9.5 determined by Nakamura et al.[22]. Thus, the substantial acidosis associated with anoxia may prevent or delay the full activation of PLA_2 even though the Ca^{2+}_i may be increased to levels that would otherwise activate the enzyme. The development of intracellular acidosis in the total renal artery clamp ischemic model in the rat may have delayed the rate of phospholipid breakdown in the study by Molitoris et al.[26]. NMR studies have shown a pronounced decrease in intracellular pH during *in vivo* ischemia[29]. Furthermore, in a less severe hypoxic model, Ca^{2+} added to the perfusate in the isolated perfused kidney model accelerates injury to the thick ascending limb and extracellular acidosis reduces the severity of that injury[30-32]. These data support the suggestion that hypoxic and anoxic injury, induced in part by phospholipase activation, may be modulated by both the ambient Ca^{2+} concentration and the degree of intracellular acidosis.

Mechanism of increases in Ca^{2+} uptake

The *in vivo* and *in vitro* protective effects of various CCB suggest that the increased rate of entry of Ca^{2+} ions into oxygen deprived renal tubules occurs via slow Ca^{2+} channels. However, this suggestion is difficult to substantiate since slow Ca^{2+} channels have not yet been identified in normal epithelial tissue. Normal epithelial tissue, including normoxic renal tubules, indeed may not have slow Ca^{2+} channels. It appears that some event, associated with the loss of K^+ and gain of Na^+ by oxygen deprived renal tubules, stimulates an increased rate of Ca^{2+} uptake. This CCB sensitive Ca^{2+} uptake pathway may be related in some manner to the decrease in membrane potential that occurs in oxygen deprivation.

Voltage-dependent Ca^{2+} channels exist in many tissues and the loss of K^+ or the decrease in membrane potential during hypoxia could explain both the increased rate of Ca^{2+} uptake, the protective effects of CCB and the lack of an effect of CCB on Ca^{2+} uptake in normal, normoxic epithelial tissues. The interpretation that changes in transmembrane ion gradients (or some result of these changes) may be the trigger for increased Ca^{2+} uptake comes from experiments in which normoxic tubules are challenged with either low extracellular Na^+ concentration which reduces the transmembrane Na^+ gradient or with high extracellular K^+ concentration, which also reduces the transmembrane K^+ gradient[33]. During oxygen deprivation the Na^+ and K^+ gradients are also reduced as cells gain Na^+ and lose K^+. Kwon et al. have shown progressive increases in Ca^{2+} uptake in normoxic rat proximal tubules as the extracellular Na^+ concentration is progressively reduced[34]. In preliminary experiments we have confirmed these data, by replacing the NaCl in normoxic bathing medium with choline chloride to maintain osmolality. These experiments were performed as control studies for experiments in which extracellular KCl concentration was raised to 40 mM with NaCl concentration lowered proportionately[35]. Oubain treatment also induces a decrease in the transmembrane Na^+ gradient[35]. The increased Ca^{2+} uptake which we observed in each of these experiments was inhibited by the CCB verapamil; specifically, verapamil normalized the Ca^{2+} uptake which would have otherwise been increased when the normoxic renal tubules were exposed to a reduced transmembrane Na^+ gradient. Frindt et al. have quantitated the extent to which membrane potential decreases under these circumstances as well as when extracellular KCl concentration in increased[33]. We have demonstrated that a reduction in the transmembrane K^+ gradient achieved by raising extracellular KCl concentration to 40 mM also leads to an increased Ca^{2+} uptake rate which is inhibited by verapamil[35]. Furthermore, amphotericin B which increases cell membrane permeability to Na^+ — thereby leading, as with ouabain, to a decreased transmembrane Na^+ gradient also causes an increased Ca^{2+} uptake that is inhibited by verapamil[35].

Although these data are provocative, similar results in oxygen-deprived tubules would be more compelling. One such study, conducted by Weinberg, showed that incubating tubules, rendered hypoxic by chemical means, in a very high K^+ medium, which would presumably delay the loss of K^+ from the cells, reduced the severity of injury during chemical hypoxia and promoted faster recovery upon drug washout[36]. Takano et al. have shown that the decrease in cellular K^+ during hypoxia is virtually complete within 10 minutes and very little further decrease seen over the next 30 minutes[11]. Weinberg and Humes have shown similar results. Specifically, the low cellular K^+ content seen at 15 minutes of hypoxia is not further altered after 30 or 60 minutes of hypoxia or anoxia[12, 13]. Thus the decrease in cellular K^+ occurs quite rapidly following an abrupt decrease in cellular ATP levels due to the absence (anoxia) or near absence (hypoxia) of oxygen.

This important observation may explain why we observed that the effect of CCB to prevent increased Ca^{2+} uptake lasts only 10 minutes during *in vitro* hypoxia in rat renal tubules[15]. By 20 minutes, a time when K^+ levels have already declined to very low levels, CCB do not prevent the increased Ca^{2+} uptake. We speculate that these results indicate that CCB are effective in preventing Ca^{2+} uptake

during hypoxia only when the transcellular K^+ gradient is *changing* acutely; this occurs during the first 10 minutes of hypoxia *in vitro* and then ceases over the next 10-50 minutes[11-13]. However, Ca^{2+} uptake remains elevated after 10 minutes of hypoxia but this later uptake is insensitive to CCB even if additional amounts of CCB are added[15]. There must, therefore, be an additional reason for the sustained Ca^{2+} uptake after 10 minutes of oxygen deprivation.

Specifically, Na^+ content of hypoxic renal tubules increases over time, roughly in a reciprocal fashion to the loss of K^+. This observation suggests that over time increase in intracellular Na^+ may activate the Na^+/Ca^{2+} exchanger, engaging it to operate in the reverse mode, to extrude Na^+ in exchange for ECF Ca^{2+}. Studies are currently underway in our laboratory to test this hypothesis. Such a mechanism, which remains highly speculative at the present time, would likely become operant only after cellular Na^+ had risen appreciably. It is likely, as Mason and coworkers suggest, that cell Na^+ reaches this critical intracellular concentration at or somewhat before 20 minutes of total ischemia *in vivo*[37]. To our knowledge Na^+/Ca^{2+} exchange is not known to be sensitive to CCB and thus our observation that Ca^{2+} uptake remains elevated during prolonged hypoxia (20 minutes) via a pathway insensitive to CCB is consistent with the activation of the putative Na^+/Ca^{2+} exchanger mechanism. This mechanism could account for the progressive, continuous accumulation of Ca^{2+} by hypoxic rabbit tubules *in vitro*[11-13]. We propose, however, that such a mechanism does not occur during prolonged anoxia due to the development of intracellular acidosis.

As mentioned above, *in vivo* infusion of CCB during reflow for either 15 minutes[4] or 1 hour[3] following ischemia in rats or dogs is not associated with functional protection. Mason et al. have reported that it takes longer than 1 hour of reflow to restore intracellular K^+ concentration following 60 minutes of renal ischemia *in vivo*[37] and Weinberg and coworkers show a delayed recovery of tissue K^+ after 30 min of *in vitro* hypoxia[12,13]. Therefore, our hypothesis, which suggests that in order to be effective CCB must be infused or present *while* the K^+ gradient is continuing to change, is consistent with the requirement for a 2-hour post-ischemic infusion in order for functional, morphologic and biochemical protection to be demonstrable. It may be, therefore, that CCB infusions will express protective effects during the time that the transmembrane K^+ gradient is either *decreasing acutely* (i.e., over the first 10 minutes of *in vitro* anoxia or hypoxia) or *increasing acutely* toward normal (during reperfusion). In addition, the rate of recovery of the transmembrane Na^+ gradient and its independent effects on Na^+/Ca^{2+} exchange may be an important contributor to the magnitude and timing of the protection induced by CCB.

Summary: Ca^{2+} ions and cell injury

The timing, pathways and degree of Ca^{2+} overload in hypoxic and anoxic renal tubules have been described above. This additional cellular Ca^{2+} burden has the propensity to cause mitochondrial Ca^{2+} overload. The majority of experimental data suggest that, dependent on the degree of intracellular acidosis, Ca^{2+}-dependent activation of phospholipases occurs. In addition, oxidant injury to mitochondria, in part related to the presence of Ca^{2+} ions, also has strong experimental support[27]. In view of this latter finding, it is not surprising that Widner and Mela-Riker have shown that verapamil attenuates oxygen deprivation injury to mitochondria[38].

Calcium ions, however, can also activate proteases and in some species, accelerate the formation of xanthine oxidase, an enzyme important for oxidant injury to renal tissue [review in 39]. Ca^{2+} ions also may lead to other changes including cytoskeletal disruption, altered membrane fluidity and separation of one cell from another at tight junctions [review in 39]. Such changes have been demonstrated to be part of the response to hypoxia and it is likely that additional derangements in other biochemical parameters will be described in the future.

The role of altered Ca^{2+} homeostasis in effecting these changes directly needs further study; however, it does appear certain that the main and early injurious events that characterize oxygen deprivation injury, are due in part to altered cellular Ca^{2+} homeostasis. These changes are initiated in response to the decrease in APT, to the subsequent loss of ionic homeostasis and to «Ca^{2+} channels» sensitive to CCB. With these observations, accumulated as a result of investigations in many laboratories over the last 10 years, it now appears that cellular Ca^{2+}, either in the cytosol (in anoxia) or mitochondria (in hypoxia), does play a major roler in the initiation of ischemic injury. CCB provide, therefore, one of the few pharmacological tools that enable investigators and clinicians to interrupt this process in a way that benefits the renal tubules *in vitro* and the whole kidney *in vitro* or *in vivo*. Many recent clinical observations appear to support the proposal that the results of these *in vivo* animal studies and *in vitro* isolated tubule studies can be applied to the patient care arena.

References

1. Schrier RW, Burke TJ, Conger JD, et al.: New aspects of acute renal failure. *Proceedings of the 8th International Congress Nephrology*, pp. 63-69. Athens: S. Karger, 1981.
2. Burke TJ, Arnold PE, Grossfeld PD, and Schrier RW: Effect of calcium membrane inhibition on norepinephrine-induced acute renal failure. In: *Acute Renal Failure*, Eliahou HE (ed.).
3. Burke TJ, Arnold PA, Gordon JA, Bulger RE, Dobyan DC, and Schrier RW: Protective effect of intrarenal calcium membrane blockers before of after renal ischemia. *J Clin Invest*, 74:1830-1841, 1984.
4. Goldfarb D, Iaina A, Serban I, Govendo S, Koupler S, and Eliahou E: Beneficial effect of verapamil in ischemic acute renal failure in the rat. *Proc Soc Exp Biol Med*, 172:389-392, 1983.
5. Wait RB, White G, and Davis JH: Beneficial effect of verapamil on post-ischemic renal failure. *Surgery*, 94:276-282, 1983.
6. Duggan KA, MacDonald GJ, and Charlesworth JA: Verapamil prevents post-transplant oliguric renal failure. *Clin Nephrol*, 24:289-291, 1985.
7. Wagner K, Albrecht S, and Neumayer HH: Prevention of delayed

graft function in cadaveric kidney transplantation by a calcium antagonist. Preliminary results of two prospective randomized trials. *Transpl Proc*, 18:510-515, 1986.

8. Malis, CD, Cheung JY, Leaf A, and Bonventre JV: Effects of verapamil in models of ischemic acute renal failure in the rat. *Amer J Physiol*, 245:F735-F742, 1983.
9. Bock HA, Brunner FP, Torthorst J, and Thiel G: Failure of verapamil to protect from ischemic renal damage. *Nephron*, 57:299-305, 1991.
10. Wilson PD, and Schrier RW: Nephron segment and calcium as determinants of anoxic cell death in renal cultures. *Kidney Int*, 29:1172-1179, 1986.
11. Takano T, Soltoff SP, Murdaugh S, and Madel LJ: Intracellular respiratory dysfunction and cell injury in short-term anoxia of rabbit renal proximal tubules. *J Clin Invest*, 76:2377-2384, 1985.
12. Weinberg JM: Oxygen deprivation-induced injury to isolated rabbit kidney tubules. *J Clin Invest*, 76:1193-1208, 1985.
13. Weinberg JM, and Humes HD: Increases of cell ATP produced by exogenous adenine nucleotides in isolated rabbit tubules. *Amer J Physiol*, 250:F720-F733, 1986.
14. Bunnachak D, Almeida ARP, Burke TJ, et al.: Time dependent phospholipase inhibition in protection against hypoxia-induced proximal tubule injury. Submitted, 1992.
15. Almeida ARP, Bunnachak D, Burnier M, et al.: Time-dependent protective effects of calcium channel blockers on anoxia- and hypoxia-induced proximal tubule injury. *J Pharm Exp Therap*, 260:526-532, 1992.
16. Weinberg JM, Davis JA, Roeser NF, and Venkatachalam MA: Role of increased cytosolic free calcium in the pathogenesis of rabbit proximal tubule cell injury and protection by glycine or acidosis. *J Clin Invest*, 87:581-590, 1991.
17. Biscoe TJ, and Duchen MR: Monitoring pO_2 by the carotid chemoreceptor. *News in Physiol Sci*, 5:229-233, 1990.
18. Lumlertgul D, Harris DCH, Burke TJ, et al.: Detrimental effect of hypophosphatemia on the severity and progression of ischemic acute renal failure. *Mineral Elect Metab*, 12:204-209, 1986.
19. Snowdowne KW, Freudenrich CC, and Borle AB: The effects of anoxia on cytosolic free calcium, calcium fluxes, and cellular ATP levels in cultured kidney cells. *J Biol Chem*, 260:11619-11626, 1985.
20. Jacobs WR, Sgamboli M, Gómez G, et al.: Role of cytosolic Ca in renal tubule damage induced by anoxia. *Amer J Physiol*, 260:C545-C554, 1991.
21. Burnier M, Van Putten VJ, Schieppati A, and Schrier RW: Effect of extracellular acidosis on $^{45}Ca^{2+}$ uptake in isolated hypoxic proximal tubules. *Amer J Physiol*, 254:C839-C846, 1988.
22. Nakamura H, Nemenoff RA, Gronich JH, and Bonventre JV: Subcellular characteristics of phospholipase A^2 activity in the rat kidney. Enhanced cytosolic, mitochondrial, and microsomal phospholipase A_2 enzymatic activity after renal ischemia and reperfusion. *J Clin Invest*, 87:1810-1818, 1991.
23. Portilla D, Mandel LJ and Bar-Sergi D, and Millington DS: Anoxia induces Phospholipase A_2 activation in rabbit proximal tubules. *Am J Physiol*, 262:F354-F360, 1992.
24. Humes HD, Nguyen VD, Cieslinski DA, and Messana JM: The role of free fatty acids in hypoxia-induced injury to renal proximal tubule cells. *Amer J Physiol*, 256:F688-F696, 1989.
25. Wetzels JFM, Wang X-N, Arnold PE, et al.: Glycine protection against hypoxic injury in isolated rat proximal tubules is not mediated by interference with activation or action of phospholipase A_2. *J Amer Soc Nephrol* (abstr.), 2:657, 1991.
26. Molitoris BA, Wilson PD, and Schrier RW: Ischemia induces partial loss of surface membrane polarity and accumulation of putative calcium ionophores. *J Clin Invest*, 76:2097-2105, 1985.
27. Malis CD, and Boventre JV: Mechanisms of calcium potentiation of oxygen free radical injury to renal mitochondria. *J Biol Chem*, 261:14201-14208, 1986.
28. Chi R, Berezesky IK, Smith MW et al.: Anoxia results in increased $[Ca^{2+}]_i$ in rat proximal tubular epithelium. *FASEB Journal* (abstr.), 6:A1060, 1992.
29. Chan L, Ledingham GG, Dixon JA, et al.: Acute renal failure: a proposed mechanism based upo ^{31}P nuclear magnetic resonance studies in the rat. In: Eliahou HE (ed.). *Acute Renal Failure*, London: John Libbey, pp. 35-41, 1982.
30. Brezis M, Shina A, Kidroni G, Epstein FH, and Rosen S: Calcium and hypoxic injury in the renal medulla of the perfused rat kidney. *Kidney Int*, 34:186-194, 1988.
31. Shanley PF, Shapiro JI, Chan L, Burke TJ, and Johnson GC: Acidosis and hypoxic medullary injury in the isolated perfused kidney. *Kidney Int*, 34:791-796, 1988.
32. Shanley PF, and Johnson GC: Calcium and acidosis in renal hypoxia. *Lab Invest*, 65:298-305, 1991.
33. Frindt G, Lee CO, Yang JM, et al.: Potential role of cytoplasmic calcium ions in the regulation of sodium transport in renal tubules. *Mineral Elect Metab*, 14:40-47, 1988.
34. Kwon S, Rothroch JK, and Domínguez JH: External sodium alters cell calcium homeostasis in rat proximal tubules. *Kidney Int* (abstr.), 37:458, 1990.
35. Schrier RW, Conger JD, and Burke TJ: Pathogenetic role of calcium in renal cell injury. In: Hatano M (ed.). *Nephrology* Tokyo: Springer-Verlag, pp. 648-659, 1991.
36. Weinberg JM, Davis JA, Abarzua M, and Kiani T: Ouabain-induced lethal proximal tubule cell injury is prevented by glycine. *Amer J Physiol*, 258:F346-F355, 1900.
37. Mason J, Beck F, Dorge A, Rick R, and Thuran K: Intracellular electrolyte composition following renal ischemia. *Kidney Int*, 20:61-70, 1981.
38. Widener LL, and Mela-Riker LM: Verapamil pretreatment preserves mitochondrial function and tissue magnesium in the ischemic kidney. *Circ Shock*, 13:27-37, 1984.
39. Burke TJ, and Schrier RW: Pathophysiology of cell ischemia. In: Schrier RW, Gottschalk CW (eds.), *Diseases of the Kidney*, 5th ed. Boston: Little, Brown and Co., 1992.