Calcium-mediated proximal tubular injury—what is the role of cysteine proteases?

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Calcium

Numerous studies over the past 15 years in different injury models and cell types have demonstrated an increase in cytosolic calcium in renal epithelial cell injury. Some of these studies are summarized in Table 1. However, despite these studies, crucial questions to implicate calcium as the primary factor in cell injury have remained. Does the increase in calcium precede the injury? Does preventing the rise in cytosolic calcium attenuate the injury? Recent studies have provided insight into these questions. Both Weinberg et al. [1] and Kribben et al. [2] conclusively demonstrated in proximal tubules that hypoxia is associated with a rise in cytosolic calcium which precedes any evidence of membrane damage. To further support a pathogenetic role of intracellular calcium, calcium-mediated injury is also reversible with reoxygenation [2], a calcium chelator to lower intracellular calcium [2] and by lowering the extracellular calcium concentration [3]. There is also compelling evidence for the role of cellular calcium in the pathophysiology of ischaemic acute renal failure (ARF) in vivo. Chemically dissimilar calcium channel blockers (CCB) are effective in preventing or attenuating the course of experimental ischaemic ARF [4]. Vascular perturbations in experimental ischaemic ARF were found to be associated with an increase in calcium in afferent arterioles from ischaemic as compared to control kidneys [5]. On the background of these experimental results, the efficacy of CCB has been shown in preventing ARF associated with cadaveric transplantation and radiocontrast media [6].

What are the mechanisms whereby increases in cytosolic free calcium lead to cell membrane injury? Potential calcium-dependent mechanisms include changes in the actin cytoskeleton of proximal tubule

Table 1. Increases in cytosolic calcium in renal epithelial cell injury

<table>
<thead>
<tr>
<th>Injury model</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ionophore</td>
<td>Rabbit proximal tubules</td>
<td>Mandel and Murphy, 1984 [21]</td>
</tr>
<tr>
<td>Anoxia</td>
<td>LLCMK2 cells</td>
<td>Snowdowne et al., 1985 [22]</td>
</tr>
<tr>
<td>Chemical ATP depletion</td>
<td>MDCK cells</td>
<td>McCoy et al., 1988 [23]</td>
</tr>
<tr>
<td>Calcium ionophore, chemical ATP depletion</td>
<td>Cultured rabbit proximal tubules</td>
<td>Phelps et al., 1989 [24]</td>
</tr>
<tr>
<td>Chemical anoxia</td>
<td>Rabbit proximal tubules</td>
<td>Weinberg et al., 1991 [1]</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Rabbit proximal tubules</td>
<td>Jacobs et al., 1991 [25]</td>
</tr>
<tr>
<td>Anoxia and hypoxia</td>
<td>Rat proximal tubules</td>
<td>Wetzsel et al., 1993 [3]</td>
</tr>
<tr>
<td>Chemical anoxia</td>
<td>Opossum kidney cells</td>
<td>Li et al., 1993 [26]</td>
</tr>
<tr>
<td>Hypoxia-reoxygenation</td>
<td>Primary culture rat proximal tubules</td>
<td>Greene and Pallor, 1994 [27]</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Rat proximal tubules</td>
<td>Kribben et al., 1994 [2]</td>
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</tbody>
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Table 2. The major groups of cysteine proteases

<table>
<thead>
<tr>
<th>Family</th>
<th>Cathepsins</th>
<th>Calpains</th>
<th>Caspases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>B,H,L,S (lysosomal)</td>
<td>μ and n calpain</td>
<td>1–13</td>
</tr>
<tr>
<td>Activation</td>
<td>Lysosome</td>
<td>Tissue specific isoforms</td>
<td>3 groups depending on substrate and function</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>Calcium-independent</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Functions</td>
<td>Intracellular protein degradation</td>
<td>Calcium-dependent</td>
<td>Caspase activated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intracellular signalling</td>
<td>7-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytoskeletal stability</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Necrosis and apoptosis</td>
<td>Cytokine activation</td>
</tr>
</tbody>
</table>

Cysteine proteases

The cysteine proteases are a group of intracellular proteases that have a cysteine residue at their active site. They consist of three major groups: cathepsins, calpains and the newly discovered caspases. The major differences between the groups of cysteine proteases are shown in Table 2. The cathepsins are non-calcium-dependent lysosomal cysteine proteases that do not appear to play a role in lethal cell injury.

Calpain, the major calcium-dependent cytosolic cysteine protease so far described and is ubiquitously present in most cell types including renal tubules. Calpain exists in the cytosol as the inactive proenzyme, procalpain, which translocates from the cytosol to the cell membrane in the presence of micromolar concentrations of cytosolic calcium. Autocatalytic activation of procalpain to active calpain occurs at the membrane in the presence of calcium and phosphatidylinositol. Activity of autolysed calpain is subject to final regulation by calpastatin, a specific endogenous calpain inhibitor.

The caspases are a newly discovered family of intracellular cysteine proteases. The term ‘caspase’ embodies two properties of these proteases in which ‘c’ refers to ‘cysteine’ and ‘aspase’ refers to their specific ability to cleave substrates after an aspartate residue. There are 13 members of the caspase family, caspases 1–13. Caspases participate in two distinct signalling pathways: (i) activation of proinflammatory cytokines and (ii) promotion of apoptotic cell death [7]. The members of the caspase family can be divided into three subfamilies based on substrate specificity and function. Caspase-1 (previously known as interleukin-1 converting enzyme or ICE) plays a major role in the activation of proinflammatory cytokines. Caspases 6, 8 and 9 are upstream components in the proteolytic cascade. These ‘initiator’ caspases pronounce the death sentence. They are activated in response to signals indicating that the cell has been stressed or damaged or has received an order to die. They clip and activate another family of caspases, the ‘executioners’ (caspases 2, 3 and 7).

Role of calpain in hypoxic proximal tubular injury

The calcium-dependent calpains have been shown to be mediators of hypoxic/ischaemic injury to brain and heart [8,9]. Calpain is also a mediator of preservation-reperfusion injury in rat liver transplantation [10]. We were first to demonstrate that calpain plays a role in hypoxia-induced necrosis to rat renal proximal tubules [11–13].

An assay for calpain in freshly isolated rat PT was developed [11]. In this study, the calcium-ionophore, ionomycin, induced a dose-dependent increase in cytosolic calcium and calpain activity. During hypoxia and ionomycin treatment, the increase in calpain activity preceded cell membrane damage. Inhibition of hypoxic-and ionomycin-induced increases in calpain activity with chemically dissimilar cysteine protease inhibitors elicited cytoprotection against accompanying PT cell membrane damage [11]. The effect of low free cytosolic calcium on this hypoxia-induced calpain activity was also determined [12]. Low free cytosolic calcium attenuated the hypoxia-induced increase in calpain activity. This attenuation of calpain activity was observed early before hypoxia-induced membrane damage and was associated with marked reduction in the typical pattern of hypoxia-induced cell membrane damage. This study suggested that the protective effect of low intracellular calcium against hypoxic injury is mediated at least in part by inhibition of calpain activity.

Role of caspases in hypoxic proximal tubular injury

The caspases, although not calcium dependent, play a key biological role in inflammation and mammalian cell death. In animal studies, caspase inhibitors are potent therapeutic agents against in vivo apoptosis. Anti-Fas antibody administered to mice leads to lethal hepatic cell apoptosis within hours, whereas mice treated with a caspase inhibitor survive and remain healthy [14]. Mice deficient in caspase-1 demonstrate...
reduced ischaemic brain injury produced by occlusion of the middle cerebral artery [15]. In addition, caspase inhibitors are effective in animal models of myocardial ischaemia-reperfusion injury. While caspases play an important role in apoptosis, they are also involved in necrotic cell death. Rat kidneys subjected to ischaemia demonstrate an increase in both caspase-1 and caspase-3 mRNA and protein expression [16].

On the basis of the dramatic protective effect of caspase inhibition on cell death in brain, heart and liver, we examined the role of caspases in hypoxia-induced necrosis in rat proximal tubules. Tubular caspase activity was increased after 15 min hypoxia in association with increased cell membrane damage as assessed by lactate dehydrogenase (LDH) release. Proximal tubules preincubated with a pancaspase inhibitor demonstrated a dose-dependent decrease in caspase activity and markedly decreased LDH release during 15 min hypoxia [17].

Next, an experiment was done to identify the caspase involved in hypoxic proximal tubular injury [17]. The caspase assay was developed using the fluorescent substrate Ac-YVAD-AMC which is preferentially cleaved by group I caspases (caspases 1, 4 and 5). The fluorescent substrate Ac-DEVD-AMC which is cleaved by group II caspases (caspases 2, 3 and 7) was also used. Caspase activity was measured in normoxic and hypoxic tubules with both substrates. Significant fluorescent activity was detected with Ac-YVAD-AMC but not with Ac-DEVD-AMC suggesting that caspases 1, 4 and 5 are involved in hypoxic injury. As a positive control, purified caspase-3 was assayed using the substrates Ac-YVAD-AMC and Ac-DEVD-AMC. Significant caspase activity was only detected with Ac-DEVD-AMC and minimal activity was detected with Ac-YVAD-AMC.

**Interaction between calpain and caspases**

Our results suggest that both calpain and caspases play a role in hypoxia-induced cell membrane damage in proximal tubules. A prelethal increase in cytosolic calcium is a cardinal feature of our hypoxic proximal tubule model. How are the non-calcium-dependent caspases activated during hypoxia? There are two possibilities. Caspase activation may be downstream of calcium-mediated activation of calpain or caspases may be activated in a separate pathway independent of calcium. Since an interaction between caspases and calpain during cell injury has been suggested [15], we studied the effect of the specific calpain inhibitor, PD150606, on the hypoxia-induced increase in caspase activity in proximal tubules [17]. PD150606 inhibits calpain activity and protects against hypoxic injury in rat proximal tubules [12]. PD150606 also attenuated the hypoxia-induced increase in caspase activity. However, PD150606 did not inhibit the activity of purified caspase-1 in vitro suggesting that calpain may be upstream of caspases during hypoxic proximal tubular injury. Next the effect of caspase inhibition on calpain activity was determined [17]. The specific caspase inhibitor, Z-D-DCB attenuated the hypoxia-induced increase in calpain activity in proximal tubules. However, Z-D-DCB did not inhibit the activity of purified calpain in vitro.

In summary, these data suggest that both caspase-mediated activation of calpain and calpain-mediated activation of caspases occur during hypoxic proximal tubular injury. These data are supported by other studies that demonstrate simultaneous activation of both calpain and caspases during cell death [18]. Thus, it is possible that during hypoxic proximal tubule injury there are different proteolytic pathways involving different caspases and calpains. A proposed relationship between caspases and calpain during hypoxic proximal tubular injury is demonstrated in Figure 1.

**Targets of caspases during hypoxic/ischaemic proximal tubular injury**

Cells that contain calpain also contain an endogenous inhibitor of calpain called calpastatin. Calpastatin, like calpain, is ubiquitously present in most cell types. The inhibitor-calpain interaction is calcium-dependent. Proteolysis of calpastatin by caspases has recently been described [18]. Thus calpastatin may be a target of caspases. The link between caspases and calpain/calpastatin is also suggested by the following. It has been reported that caspase-1 (ICE) knockout mice have decreased levels of both IL-1β and IL-1α [15]. It is well established that, while caspase-1 is the pro-IL-1β processing enzyme, calpain is the preferred IL-1α processing enzyme. This decrease in the calpain-mediated production of IL-1α in caspase-1 knockout mice is intriguing. It is possible that caspase-1 may activate calpain either directly or via calpastatin degradation.

The interaction between caspases and calpastatin was investigated during renal ischaemia-reperfusion injury [19]. Renal ischaemia-reperfusion injury was

![Fig. 1. Hypoxia-induced necrosis in proximal tubules may involve activation of proteolytic pathways involving different calpains and caspases.](image-url)
induced by renal pedicle clamp for 45 min followed by 6 h reperfusion. Immunoblots were performed on renal cortex with a monoclonal calpastatin antibody. A low-molecular weight (LMW) form of calpastatin was detected in control rat kidney cortex. There was a decrease in this LMW calpastatin after ischemia-reperfusion. To determine whether the decreased protein expression had functional significance, calpastatin activity was measured. Calpastatin activity decreased significantly after ischemia-reperfusion compared to sham-operated controls. Calpastatin activity normalized in a group of rats pretreated with a pancaspase inhibitor before ischemia-reperfusion. The increase in calpastatin activity by caspase inhibition suggested that caspases may be proteolyzing calpastatin. Caspase-3 activity measured in renal cortex increased significantly after ischemia-reperfusion compared to sham-operated controls. These preliminary data suggest that proteolysis of calpastatin by caspase-3 may regulate calpain activity during ischemia-reperfusion injury. In this regard, an increase in calpain activity as determined by (i) the appearance of calpain-mediated spectrin breakdown products and (ii) the conversion of procalpain to active calpain, has been demonstrated during renal ischemia-reperfusion injury [20].

Conclusion

Both calpain and caspases are mediators of hypoxic injury in proximal tubules. Inhibitor studies demonstrate an interaction between these two cysteine proteases suggesting that hypoxia-induced necrosis in proximal tubules may involve activation of proteolytic pathways involving different calpains and caspases (Figure 1). On the background of the dramatic protective effect of caspase inhibition on cell death in brain, heart and liver and the role of caspases as mediators of renal proximal tubular injury, studies are being undertaken to determine the role of caspases in ischemic acute renal failure in vivo.

References
