Partial ATP depletion induces Fas- and caspase-mediated apoptosis in MDCK cells

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Partial ATP depletion induces Fas- and caspase-mediated apoptosis in MDCK cells. Am. J. Physiol. 276 (Renal Physiol. 45): F837–F846, 1999.—Brief periods of in vitro hypoxia/ischemia induce apoptosis of cultured renal epithelial cells, but the underlying mechanisms remain unknown. We show that partial ATP depletion (∼10–65% of control) results in a duration-dependent induction of apoptosis in Madin-Darby canine kidney (MDCK) cells, as evidenced by internucleosomal DNA cleavage (DNA laddering and in situ nick end labeling), morphological changes (cell shrinkage), and plasma membrane alterations (externalization of phosphatidylserine). The ATP-depleted cells display a significant upregulation of Fas, Fas ligand, and the Fas-associating protein with death domain (FADD). Exogenous application of stimulatory Fas monoclonal antibodies also induces apoptosis in nonischemic MDCK cells, indicating that they retain Fas-dependent pathways of programmed cell death. Furthermore, cleavage of poly(ADP)ribose polymerase (PARP) is evident after ATP depletion, indicating activation of caspases. Indeed, the apoptotic cells display a significant increase in caspase-8 (FLICE) activity. Finally, apoptosis induced by ATP depletion is ameliorated by pretreatment with inhibitors of caspase-8 (IETD), caspase-1 (YVAD), or caspase-3 (DEVD) but is not affected by inhibitors of serine proteases (TPCK). Our results indicate that partial ATP depletion of MDCK cells results in apoptosis and that Fas- and caspase-mediated pathways may play a critical role.

Apoptosis, or programmed cell death, is characterized by distinct morphological changes consisting of cell shrinkage, nuclear condensation, and internucleosomal DNA fragmentation (19) and has been shown to play a significant role in the normal development of the kidney (20, 21). Apoptosis has also been observed in an increasing array of renal disorders (4, 23, 34) and particularly mediates renal tubule cell death in autosomal dominant and recessive polycystic kidney disease (41), ureteral obstruction (6), and renal transplant rejection (27). Perhaps most significantly, brief periods of in vivo renal ischemia are consistently associated with apoptosis of tubule cells (29, 33, 35, 43, 44). Induction of apoptosis in these situations may be associated with the activation of stress kinases and endonucleases (39, 42). The purpose of apoptosis in renal ischemia is controversial and intriguing. On the one hand, apoptosis during the late recovery period may be an important mechanism by which previously damaged cells are removed, allowing for tubule regeneration (36). Alternatively, apoptosis during the early periods of ischemia/reflow may represent a direct pathogenic mechanism by which tubule cells are damaged after an ischemic insult, and downregulation of apoptosis may offer a unique and powerful therapeutic approach to the amelioration of ischemic renal injury (1, 33, 39). This possibility has contributed to the interest in characterizing apoptosis in renal epithelial cells in vitro. Indeed, ischemic injury produced by intracellular ATP depletion has recently been shown to induce apoptosis in LLC-PK1 (porcine proximal tubule) cells (13–15) and in primary cultures of mouse proximal tubule cells (24). However, the mechanisms and pathways involved in the stimulus recognition and signal transduction leading to renal cell apoptosis after ischemic injury or ATP depletion are unknown.

The most thoroughly studied (and most physiologically relevant) apoptotic pathways result from Fas- or tumor necrosis factor (TNF) receptor-dependent protein-protein interactions, facilitated by molecules that possess a conserved “death domain” (11, 28, 40). Both Fas receptors and TNF receptors are integral membrane proteins that possess the characteristic death domain, enjoy a wide tissue distribution, and participate in the following two models for the induction of apoptosis in a variety of cell types. In the first model, an activated Fas receptor binds the Fas-associating protein with death domain (FADD), with resultant activation of caspase-8 (FLICE). This pathway can be stimulated in vitro by monoclonal antibodies to Fas (8). In the second model, activated TNF receptor 1 (TNFR1) interacts with TRADD (TNFR1-associated death domain protein), which, in turn, recruits FADD. Both pathways can stimulate the caspase-1 family (ICE) of proteases (2, 7) and finally result in the activation of caspase-3 (CPP32/Yama/Apopain). Caspase-3 cleaves several substrates, including poly(ADP)ribose polymerase (PARP), lamins, and actins, with resultant chromosomal DNA degradation and cellular morphological changes characteristic of apoptosis. Promising new findings suggest that inhibition of caspases protects against hypoxia/reperfusion-induced apoptosis in neurons (16), endothelial cells (17), and hepatocytes (37).

In this study, we have modified a previously described protocol of in vitro ischemia (3, 5, 23, 25, 38) to...
achieve graded levels of ATP depletion in Madin-Darby canine kidney (MDCK) collecting tubule cells. We show that complete ATP depletion (<5% of control) results in necrosis. Partial ATP depletion (10–65% of control) induces apoptosis, as evidenced by internucleosomal DNA cleavage, changes in cellular morphology, and alterations in the plasma membrane. This study demonstrates, for the first time, that partial ATP depletion also results in a significant upregulation of at least three molecules that have been implicated in the early stimulus detection and transduction phases of the apoptotic process in other cell types, namely Fas, Fas ligand, and FADD. Exogenous application of stimulatory Fas monoclonal antibodies also induces programmed cell death in MDCK cells not depleted of ATP, indicating that they retain Fas-dependent apoptotic pathways. Furthermore, cleavage of PARP is evident after partial ATP depletion, indicating the activation of caspsases. Indeed, the apoptotic cells display a significant increase in caspase-8 (FLICE) activity. Finally, apoptosis induced by ATP depletion is ameliorated by pretreatment with inhibitors of caspase-8 (IETD), caspase-1 (YVAD), or caspase-3 (DEVD) but is not affected by inhibitors of serine proteases (TPCK). Our results indicate that partial ATP depletion of MDCK cells results in apoptosis and that the Fas-FADD axis and caspases (specifically caspase-8) may play a critical role in the process.

**METHODS**

Cell culture. MDCK type II cells, obtained from American Type Culture Collection (Manassas, VA), were passaged in DMEM with 10% fetal bovine serum ( Gibco BRL, Gaithersburg, MD) and analyzed within 1 to 2 days of reaching confluence.

ATP depletion. We modified previously described protocols (3, 5, 24, 25, 38) to achieve graded levels of ATP depletion in MDCK cells. The first set of experiments utilized a combination of glycolytic (2-deoxy-D-glucose; Sigma, St. Louis, MO) and oxidative (antimycin A; Sigma) inhibitors. Confluent monolayers of MDCK cells grown on six-well tissue culture-treated polystyrene plates (Corning, NY) were washed with PBS and incubated in Dulbecco’s PBS with 1.5 mM CaCl₂, 2 mM MgCl₂, 10 µM antimycin A, and 2 mM 2-deoxy-o-glucose for various periods of time. For microscopy, cells were grown directly on coverslips placed within the six-well plates. Control cells were incubated in regular medium. ATP measurements were performed with a luciferase-based assay kit (Sigma). Briefly, any nonadherent cells were pelleted, washed with PBS, and solubilized in 500 µl of somatic cell ATP-releasing agent, and the mixture was added to the washed adherent cells. After a 5-min incubation period, the entire sample was cleared of insoluble material by centrifugation, and equal volumes of the supernatant were added to an equal volume of ATP assay mix. ATP levels were measured with a luminometer and expressed as a percentage of control values.

In preliminary experiments, we noted that inhibition of both glycolysis and oxidative phosphorylation resulted in a profound depletion of ATP levels and subsequent necrotic cell death. AA alone yielded duration-dependent partial depletion of ATP, which was partially reversible during recovery.

Other experimental conditions. To examine the effects of Fas and TNFα activation, for 4 or 8 h, we exposed confluent MDCK cells to stimulatory Fas monoclonal antibody (clone DX2; Clontech, La Jolla, CA) at 200 ng/ml, human TNFα (Clontech) at 200 ng/ml, cycloheximide (Sigma) at 10 µg/ml, or a combination of TNFα and cycloheximide. To assess the role of caspases and serine proteases, we pretreated cells for 2 h with an inhibitor of caspase-1 (ICE), YVAD-cmk (50 µM; Clontech); an inhibitor of caspase-8 (FLICE), IETD-fmk (10–50 µM; Clontech); an inhibitor of caspase-3 (CPP32/Yama/Apoptain), DEVD-CMK (50 µM; Clontech); or with the serine protease inhibitor, TPCK (100 µM; Sigma). Cells were then subjected to 8 h of partial ATP depletion with Dulbecco’s PBS containing 1.5 mM CaCl₂, 2 mM MgCl₂, and 10 µM antimycin A in the continued presence of the respective caspase inhibitor.

Apoptosis assays. Internucleosomal DNA fragmentation was detected primarily by DNA laddering (31). Briefly, nonadherent cells were pelleted, washed with PBS, and added to washed and scraped adherent cells. All cells were pelleted, resuspended in 500 µl of lysis buffer (1% SDS, 25 mM EDTA, and 1 mg/ml proteinase K, pH 8), and incubated overnight at 50°C. Ribonuclease A (10 mg/ml) was then added, for an additional 2-h incubation at 37°C. The chromosomal DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed by agarose gel electrophoresis followed by staining with ethidium bromide to reveal the fragmentation pattern.

We confirmed DNA fragmentation in situ utilizing the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (ApoAlert DNA Fragmentation Assay Kit; Clontech), by which fluorescein-dUTP incorpo-
ration at the free ends of fragmented DNA can be visualized by fluorescent microscopy (12, 32). Briefly, adherent cells, grown on coverslips and subjected to experimental conditions as above, were washed with PBS, fixed with 4% formaldehyde/PBS for 30 min at 4°C, permeabilized with 0.2% Triton X-100/PBS for 15 min at 4°C, and incubated with a mixture of nucleotides and TdT enzyme for 60 min at 37°C in a dark, humidified incubator. The reaction was terminated with 2× SSC, the cells were washed with PBS, and the coverslips were mounted on glass slides with Crystal/mount (Biomeda, Foster City, CA). Fluorescent nuclei were detected by visualization with a microscope equipped with fluorescein filters (IX70; Olympus).

Because internucleosomal DNA cleavage may also be observed in necrotic cells, it was important to confirm the occurrence of apoptosis by additional assays (9, 23, 24). The characteristic morphological changes of apoptosis, including cell shrinkage and blebbing, were detected by direct light microscopy (24, 31). Because nuclear condensation is often seen during ATP depletion (independent of apoptosis), this morphological criterion was not used in this study.

In addition, we used the annexin V-FITC cell membrane labeling assay (ApoAlert Annexin V Kit; Clontech) to detect translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface, where it binds an annexin V-FITC conjugate and serves as an early marker of apoptosis (26). Nonadherent cells, or cells grown on coverslips, were incubated with annexin V-FITC for 15 min at room temperature in the dark, and visualized by fluorescent microscopy with fluorescein filters, as before. In some cases, the cells were also stained with propidium iodide (Clontech) and visualized using rhodamine filters.

Other methods. For SDS-PAGE, both adherent and nonadherent cells were washed with PBS, solubilized in 2× SDS sample buffer, and boiled for 10 min. Monoclonal antibodies to Fas, Fas ligand, and FADD used for Western analysis were from Transduction Laboratories, and the PARP monoclonal antibody was from Clontech. Immunodetection of transferred proteins was done by enhanced chemiluminescence (Amer sham). We detected caspase-8 activity using a fluorescent assay kit (Clontech).

RESULTS

Metabolic inhibition results in a duration-dependent depletion of intracellular ATP. Confluent MDCK cells subjected to inhibition of glycolysis (with 2-deoxy-D-glucose) and oxidative phosphorylation (antimycin A) displayed a rapid and profound decrease in intracellular ATP levels to <4% of controls within 2 h (Fig. 1; AA + deoxyglucose) and showed signs of necrotic death within 6 h of treatment (data not shown). Others have reported similarly severe ATP depletion under these conditions (38). However, cells subjected to only antimycin A showed a duration-dependent, partial depletion of intracellular ATP (Fig. 1; AA only). Thus ATP levels were 28 ± 4.7%, 15.5 ± 1.6%, and 9.6 ± 1.5% of controls (means ± SD from 3 separate experiments) at 2, 4, and 6 h, respectively. When cells are previously subjected to 4 h of partial ATP depletion were then allowed to recover in regular medium, a significant elevation of ATP levels was evident, to 48.7 ± 3.3% and 65.3 ± 4.1% of controls (means ± SD from 3 separate experiments) at 4 and 8 h of recovery, respectively (Fig. 1; AA + recovery). Thus a spectrum of intracellular ATP depletion from ~10–65% of control was reproducibly achieved, depending on the duration of antimycin A treatment and the period of recovery.

Partial ATP depletion induces apoptosis. We performed a series of apoptosis assays on confluent MDCK cells subjected to varying degrees of intracellular ATP depletion. Approximately 80% and 60% of cells remained adherent after 6 and 8–12 h of ATP depletion, respectively. Both adherent and nonadherent cells displayed evidence of activation of programmed cell death. Internucleosomal DNA fragmentation was detectable by the characteristic 180-bp laddering pattern in cells subjected to 6 h of ATP depletion, and was obvious after 8–12 h of ATP depletion or after 4 h of ATP depletion and either 4 or 8 h of recovery (Fig. 2). Because DNA laddering is a late indicator of apoptosis, these results suggest that the apoptotic cascade was activated during the early hours of treatment, when ATP depletion was only partial.

To test the hypothesis that short periods of partial ATP depletion can activate programmed cell death, we examined MDCK cells for an early marker, namely the translocation of phosphatidylinerine from the inner surface of the plasma membrane to the cell surface, where it binds an annexin V-FITC conjugate. Indeed, after only 4 h of ATP depletion, 40 ± 11% of adherent MDCK cells (means ± SD from 3 separate experiments) displayed a prominent annexin staining at the plasma membrane, as shown in Fig. 3A. The majority of such annexin-positive adherent cells excluded propidium iodide, indicating preservation of plasma membrane integrity and absence of necrosis (23, 24). Even at this early stage of partial ATP depletion, all the nonadherent cells were positive for annexin, and many displayed the characteristic morphology of apoptotic cells, including cell shrinkage and membrane blebbing (Fig. 3B). After 6 h of ATP depletion, 67 ± 10% of adherent MDCK cells (means ± SD from 3 separate experiments) were annexin positive, but continued to exclude propidium iodide. In addition, these cells were now positive for DNA fragmentation, as shown by the in situ TUNEL assay (Fig. 3A). After 8 h of ATP depletion, the cells were noted to be propidium iodide positive, indicating a loss of plasma membrane integrity and the possible onset of necrotic cell death.

Partial ATP depletion results in upregulation of Fas, Fas ligand, and FADD, and cleavage of PARP. Because the Fas-FADD axis is thought to be an important mechanism for the recognition and transduction of apoptotic stimuli, it was of interest to determine whether these molecules may participate in the programmed cell death induced by ATP depletion. Confluent MDCK cells subjected to varying periods of partial ATP depletion were analyzed by SDS-PAGE and Western blotting with monoclonal antibodies against Fas, Fas ligand, or FADD. All three proteins were detected in low abundance in control MDCK cells, and were significantly upregulated after 4 or 6 h of partial ATP depletion (Fas at 45 kDa, Fas ligand at 37 kDa, and FADD at 24 kDa; Fig. 4).

Because the Fas-FADD pathway results in activation of caspases, with subsequent cleavage of substrates
such as PARP, it was important to determine whether caspases were also activated after partial ATP depletion. In control MDCK cells, PARP was detected predominantly as the full-length, 116-kDa peptide, with a small amount of the 85-kDa proteolyzed fragment (Fig. 4). Cells subjected to 6 h of partial ATP depletion exhibited a significant decrease in the 116-kDa parent peptide. An additional proteolytic product was consistently detected at 55 kDa after both 4 and 6 h of partial ATP depletion, followed by either 4 or 8 h of recovery, also displayed apoptosis (lanes K and L, respectively). Because DNA laddering is a late manifestation of programmed cell death, these results suggest that apoptotic cascade was activated during early hours of treatment, when ATP depletion was only partial.

MDCK cells retain Fas-dependent apoptotic pathways. Given the above results, it was important to establish that MDCK cells retain pathways required for Fas-mediated apoptosis. Application of stimulatory monoclonal Fas antibodies induced apoptosis in cells not depleted of ATP, as detected by positive annexin V staining after 4 h, and by characteristic DNA laddering, as well as by in situ TUNEL assay after 8 h of treatment (Fig. 5). Interestingly, MDCK cells were relatively resistant to the proapoptotic properties of either TNF-α or cycloheximide alone. Programmed cell death could be induced only by a combination of TNF-α and cycloheximide, suggesting that Fas-dependent pathways of apoptosis may predominate in this cell line.

Partial ATP depletion results in stimulation of caspase-8 activity. Because partial ATP depletion of MDCK cells results in upregulation of the Fas-FADD axis, it was of interest to examine the activity of caspase-8 (FLICE), which is the most proximal member of the caspase family that is activated in Fas-dependent apoptotic pathways. Indeed, we detected an increase in specific caspase-8 activity within 2 h of ATP depletion, as determined by fluorescent assays (Fig. 6). This increase in caspase-8 activity was blocked by preincubation of cells for 2 h with 10 µM IETD-fmk peptide, a specific inhibitor of caspase-8 (Fig. 6).

Caspase inhibitors ameliorate apoptosis induced by partial ATP depletion. Because our results indicated that partial ATP depletion in MDCK cells induced apoptosis via caspase activation, it was of significant interest to examine the effects of caspase inhibition. Pretreatment and maintenance of cells with inhibitors of caspase-8 (IETD-fmk), caspase-1 (YVAD-cmk), or caspase-3 (DEVD-CHO) significantly ameliorated the DNA laddering typically seen after 8 h of ATP depletion, whereas the serine protease inhibitor TPCK did not have a protective effect (Fig. 7).
Fig. 3. Partial ATP depletion induces apoptosis. A: confluent MDCK cells, grown on coverslips, were subjected to varying periods of ATP depletion with antimycin A. At each time interval, we stained 1 set of cells with both annexin and propidium iodide (PI), and a second set of cells using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) kit. After 4 h of partial ATP depletion, cells were annexin positive but excluded PI, indicating early apoptosis but absence of necrosis. After 6 h, cells continued to be annexin positive and PI negative, but were also TUNEL positive, indicating DNA fragmentation characteristic of apoptosis. After prolonged ATP depletion (8 h), cells lost plasma membrane integrity and become PI positive. CON, untreated control cells. B: after 4 h of ATP depletion in confluent MDCK cells, ~15% of cells become nonadherent. These cells display characteristic morphology of apoptotic cells, including cell shrinkage and membrane blebbing (top, phase) and are uniformly positive for annexin staining (bottom, immunofluorescence). Bar, 5 µm.
DISCUSSION

These studies show that in vitro ischemia induced by partial ATP depletion results in apoptosis of MDCK cells, according to morphological, biochemical, and molecular criteria. We have demonstrated, for the first time, that this is associated with a marked overexpression of Fas, Fas ligand, and FADD, and cleavage of PARP, indicating activation of caspases. Stimulatory
Fig. 5. MDCK cells retain Fas-dependent apoptotic pathways. A: chromosomal DNA was extracted from MDCK cells treated for 8 h with stimulatory Fas monoclonal antibody at 200 ng/ml (Fas), tumor necrosis factor-α at 200 ng/ml (TNF), cycloheximide at 10 µg/ml (CHX), or a combination (TNF + CHX) and was analyzed by agarose gel electrophoresis. Lane M contains 100-bp DNA marker. Characteristic 180-bp laddering of internucleosomal DNA fragmentation was seen in cells treated with Fas, or with a combination of TNF-α and CHX. B: MDCK cells treated for 4 h with Fas or TNF-CHX and stained with annexin V (top) revealed staining at plasma membrane, representative of early apoptosis. MDCK cells similarly treated (but for 8 h) and analyzed by TUNEL assay (bottom) showed condensed and fluorescent nuclei, characteristic of apoptotic cells. Untreated control cells (CON) were largely negative for annexin and TUNEL staining. Bars, 5 µM.
Fas monoclonal antibodies also induced programmed cell death in MDCK cells not depleted of ATP, confirming the presence of Fas-dependent apoptotic pathways. We have also shown, for the first time, that apoptosis induced by partial ATP depletion is accompanied by increased activity of caspase-8 and is ameliorated by pretreatment with inhibitors of caspase-8. These results indicate that Fas- and caspase-mediated pathways may play a critical role in the apoptosis induced by partial ATP depletion in MDCK cells.

Renal tubule cell apoptosis has been consistently observed after brief periods of in vivo renal ischemia (29, 33, 43, 44) and may represent a direct mechanism by which tubule cells are damaged. Inhibition of apoptosis may, therefore, offer a unique approach to the amelioration of ischemic renal injury (1, 33, 39). This possibility has stimulated interest in the characterization of renal epithelial cell apoptosis in vitro. Indeed, ischemic injury produced by intracellular ATP depletion has recently been shown to induce apoptosis in LLC-PK1 cells (14–16) and primary cultures of mouse proximal tubule cells (24). The latter study elegantly demonstrated that proximal tubule cells subjected to partial ATP depletion (≤25–70% of control) died by apoptosis, whereas ATP depletion below 15% of control resulted in necrosis. Our findings in MDCK cells are in agreement with that study and emphasize the emerging concepts that 1) apoptosis is a process that requires energy and 2) intracellular ATP concentration plays a crucial role in the determination of cell death fate by apoptosis or necrosis (10, 22). In Jurkat cells, ATP has been shown to be required for apoptotic signal transduction, both upstream and downstream of caspase activation, although it appears to be most crucial to the final DNA fragmentation stage (10, 22).

Although activation of endonucleases (39) has been implicated in the final postmortem phase (40), the proximal pathways involved in the stimulus recognition, signal transduction, and effector phases of renal tubule cell apoptosis after ischemic injury or ATP depletion are largely unknown. Because both Fas expression and apoptosis have been shown to increase in mouse renal tubule cells after endotoxin treatment (30), we examined the role of Fas in MDCK cell apoptosis resulting from partial ATP depletion. We have shown, for the first time, a significant upregulation of Fas early in in vitro ischemic injury and a concomitant increase in Fas ligand and FADD expression. Furthermore, we have shown that Fas alone was pro-apoptotic in nonischemic MDCK cells, indicating that Fas-dependent pathways of apoptosis are present in this cell line. TNFR1, the other well-known receptor for apoptotic stimuli, does not appear to play a major role because MDCK cells were relatively resistant to TNF-α alone. Similar results have recently been reported for LLC-PK1 cells, where the apoptotic effects of TNF-α were pronounced only in the presence of cycloheximide (31). Taken together, our data suggest that death domain-containing proteins such as those implicated in the Fas-FADD cascade (28) participate in the apoptotic signal transduction after ATP depletion of MDCK cells.

Because the Fas-FADD cascades are thought to sequentially activate the caspase-8, caspase-1, and caspase-3 families of proteases, we sought evidence for...
such activation. Indeed, partial ATP depletion resulted in the cleavage of PARP to its proteolyzed products, a phenomenon that is well known to result from caspase-3 activation (7). Furthermore, we have demonstrated, for the first time, a significant and specific increase in caspase-8 activity in MDCK cells after partial ATP depletion. Pretreatment of MDCK cells with inhibitors of caspase-8 resulted in a marked amelioration of apoptosis induced by partial ATP depletion. Inhibitors of caspase-1 and caspase-3 were also effective in preventing apoptosis of MDCK cells after partial ATP depletion. Similar results have recently been reported in cultured LLC-PK1 cells (18). In contrast, pretreatment with serine protease inhibitors was not effective in preventing apoptosis. Caspase inhibitors have also recently been shown to protect from hypoxia-induced apoptosis in neurons (16), endothelial cells (17), and hepatocytes (37). Thus our results lend support to the notion that inhibition of apoptosis may offer a novel approach to cytoprotection of a variety of cell types from hypoxic-ischemic injury.

In summary, we have demonstrated that partial ATP depletion induces apoptosis via Fas- and caspase-dependent pathways. It will be important to confirm the role of the Fas-FADD axis and caspases in apoptosis after in vivo renal ischemia, to identify other death domain-containing molecules that may also be cooperatively involved, and to identify other factors that may have an inhibitory effect (such as Bcl-2 and growth factors). A better understanding of such stimulatory and inhibitory influences on renal tubule cell apoptosis may reveal clues for the rational design of novel therapeutic interventions for ischemic renal injury.

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