Acidic pH inhibits ATP depletion-induced tubular cell apoptosis by blocking caspase-9 activation in apoptosome

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Acidic pH inhibits ATP depletion-induced tubular cell apoptosis by blocking caspase-9 activation in apoptosome. Am J Physiol Renal Physiol 289: F410–F419, 2005. First published March 8, 2005; doi:10.1152/ajprenal.00440.2004.—Tubular cell apoptosis has been implicated in the development of ischemic renal failure. In in vitro models, ATP depletion-induced apoptosis of tubular cells is mediated by the intrinsic pathway involving Bax translocation, cytochrome c release, and caspase activation. While the apoptotic cascade has been delineated, much less is known about its regulation. The current study has examined the regulation of ATP depletion-induced tubular cell apoptosis by acidic pH, a common feature of tissue ischemia. Cultured renal tubular cells were subjected to 3 h of ATP depletion with azide and then recovered in full culture medium. The treatment led to apoptosis in ~40% of cells. Apoptosis was significantly reduced, if the pH of ATP depletion buffer was lowered from 7–7.4 to 6–6.5. This was accompanied by the inhibition of caspase activation. However, acidic pH did not prevent Bax translocation and oligomerization in mitochondria. Cytochrome c release from mitochondria was not blocked either, suggesting that acidic pH inhibited apoptosis at the postmitochondrial level. To determine the postmitochondrial events that were blocked by acidic pH, we conducted in vitro reconstitution experiments. Exogenous cytochrome c, when added into isolated cell cytosol, induced caspase activation. Such activation was abrogated, when pH during the reconstitution was lowered to 6 or 6.5. Nevertheless, acidic pH did not prevent the recruitment and association of caspase-9 by Apaf-1, as shown by coimmunoprecipitation. Together, this study demonstrated the inhibition of tubular cell apoptosis following ATP depletion by acidic pH. A critical step blocked by acidic pH seems to be caspase-9 activation in apoptosome.

ATP depletion; ischemia

Two major pathways of apoptosis have been delineated. In the extrinsic pathway, ligation of death receptors leads to the formation of a death-inducing signaling complex and the activation of caspase-8 (1). In the intrinsic pathway, cellular stress leads to mitochondrial disruption, releasing apoptogenic molecules including cytochrome c (7). Cytochrome c, after being released from mitochondria, binds Apaf-1 in the cytosol, recruiting caspase-9 to form the caspase activation complex called apoptosome. Both apoptotic pathways have been implicated in the development of ischemic renal injury and renal failure (9, 28).

In vitro models, our previous work suggested a critical role for the intrinsic pathway in tubular cell apoptosis following renal cell hypoxia or ATP depletion (14, 15, 31, 44). Under the experimental conditions, Bax, a proapoptotic Bcl-2 family protein, translocated to mitochondria and oligomerized in the outer membrane. Consequently, cytochrome c was released from mitochondria, followed by the activation of caspases in the cytosol and development of apoptotic morphology (14, 15, 31, 44). These observations have been confirmed and extended in related models of tubular cell apoptosis in vitro (12, 25) and in vivo in ischemia-reperfused cadaveric kidney allografts (9). Our recent work selected death-resistant tubular cells through repeated episodes of hypoxia (14). The cells upregulated the antiapoptotic protein Bcl-XL, which prevented Bax activation and cytochrome c release, resulting in the preservation of cell viability (14). These findings further support a role for the intrinsic pathway in tubular cell apoptosis.

While the apoptotic events at the mitochondrial and postmitochondrial levels have been delineated, much less is known about their regulation by cytosolic factors. Nevertheless, alterations of cellular pH have been demonstrated during apoptosis (22, 38). Importantly, in different experimental models, the pH changes seem essential for the initiation and progression of apoptosis. For example, in staurosporine-induced apoptosis of HeLa cells, there was a rise of intracellular pH, whereas tumor necrosis factor-α-induced apoptosis was accompanied by a drop in pH. In both models, prevention of pH changes inhibited apoptosis by blocking critical mitochondrial events including Bax activation and cytochrome c release (38). Recent studies by Segal and colleagues (4, 34) further demonstrated that alkaline pH inhibited caspase activation in an in vitro system by blocking apoptosome maturation. In the current study, we determined the effects of acidic pH on ATP depletion-induced apoptosis (10, 20, 21, 23, 35, 39, 41).

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apoptosis in renal tubular cells. We were particularly interested in acidic pH, because acidosis is a common feature of ischemic injury in organs including the brain, heart, and kidneys (5, 24, 36). We show that acidic pH inhibits tubular cell apoptosis following ATP depletion. However, mitochondrial events of apoptosis including Bax activation and cytochrome c release are not affected. A critical step that is blocked by acidic pH seems to be caspase-9 activation in apoptosome.

MATERIALS AND METHODS

Materials. Rat kidney proximal tubular epithelial cells (RPTC) were originally obtained from Dr. U. Hopfer at Case Western Reserve University, Cleveland, OH. The cells were cultured as described previously (45). HK-2 cells were purchased from ATCC (Manassas, VA) and cultured according to the instruction. DEVD.AFC and free APC were purchased from Enzyme Systems Products (Dublin, CA). Dithiobis (sucinimidyl propionate) (DSP) was purchased from Pierce (Rockford, IL). The rabbit polyclonal antibody specific to the active form of caspase-3 was a gift from Dr. A. Srinivasan at Idun Pharmaceuticals (La Jolla, CA). Other antibodies were purchased from the following sources: polyclonal antibody to lamin B from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibody to cytochrome c from BD Pharmingen (San Diego, CA); monoclonal antibody to Bax from NeoMarkers (Fremont, CA); monoclonal antibody to caspase-9 from R&D Systems (Minneapolis, MN); monoclonal antibody to Apaf-1 from Cell Signaling Technologies (Beverly, MA); and all secondary antibodies from Jackson ImmunoResearch (West Grove, PA). Other reagents were purchased from Sigma (St. Louis, MO).

ATP depletion. ATP depletion was conducted as detailed recently (15, 43, 44). Briefly, cells were incubated with 10 mM azide for 3 h in glucose-free Krebs-Ringer bicarbonate solution (composition in mM: 115 NaCl, 3.5 KCl, 25 NaHCO3, 1 KH2PO4, 1.25 CaCl2, and 1 MgSO4; gassed with 5% CO2). The pH of the solution was monitored before, during, and after ATP depletion. After ATP depletion, groups of cells were transferred to full culture medium for recovery.

Morphological examination of apoptosis. Apoptosis was examined by morphological criteria as described previously (14, 41, 44). Cell morphology was monitored by phase contrast microscopy. Nuclear morphology was examined by fluorescence microscopy after Hoechst 33342 staining. Typical apoptotic cells showed cellular shrinkage, formation of apoptotic bodies, nuclear condensation, and fragmentation.

Measurement of caspase activity. Caspase activity was measured enzymatically using the fluorogenic peptide substrate DEVD.AFC (13, 14, 41, 44). Briefly, cells were extracted with 1% Triton X-100. The lysates of 25 μg protein were added to enzymatic reactions containing 50 μM DEVD.AFC. After 60 min of reaction, fluorescence at excitation 360 nm/emission 530 nm was monitored by a GENios plate-reader (Tecan US, Research Triangle Park, NC). For each measurement, a standard curve was constructed using free AFC. Based on the standard curve, the fluorescence reading from each enzymatic reaction was converted into the nanomolar amount of liberated AFC to indicate caspase activity.

Immunofluorescence of cytochrome c, Bax, and active caspase-3. Cells were grown on collagen-coated glass coverslips for immunofluorescence as described in our previous work (14, 16, 44). For immunofluorescence of Bax and active caspase-3, cells were fixed with 4% paraformaldehyde. For immunofluorescence of cytochrome c, cells were fixed with a modified Zamboni’s fixative containing picric acid and 4% paraformaldehyde. The fixed cells were incubated with 2% normal goat serum for blocking and then exposed to specific primary antibodies (rabbit polyclonal anti-active caspase-3, mouse monoclonal anti-Bax, or mouse monoclonal anti-cytochrome c). Finally, the cells were incubated with Cy3-labeled goat anti-rabbit, Cy3-labeled goat anti-mouse, or FITC-labeled goat anti-mouse secondary antibodies. Signals were examined by fluorescence microscopy using Cy3 or FITC channel.

Cellular fractionation. To analyze the subcellular redistributions of Bax and cytochrome c during apoptosis, cells were fractionated into cytosolic fraction and the membrane-bound organellar fraction. The fractionation was facilitated by using low concentrations of digitonin, as described in our previous work (14, 16, 31, 41). Digitonin at low concentrations selectively permeabilizes the plasma membrane, without solubilizing mitochondria. The feasibility of digitonin extraction in our experiments was further supported by two observations: first, digitonin did not extract or solubilize cytochrome c from mitochondria in normal control cells, where cytochrome c resided in the intermembrane space of the organelles (see Fig. 3A); second, GOX IV (an integral mitochondrial protein) was not detected in digitonin-soluble fraction, suggesting that contamination of this fraction by mitochondria was indeed minimal (data not shown). Cellular fractionation by digitonin was also utilized by other investigators to examine protein translocations during apoptosis (29, 42). Briefly, cells were incubated with 0.05% digitonin in isotonic sucrose buffer (in mM: 250 sucrose, 10 HEPES, 1 KCl, 1.5 MgCl2, 1 EDTA, and 1 EGTA; pH 7.1) for 2 min at room temperature. The released cytosol was collected. The digitonin-insoluble part was further extracted with 2% SDS to collect the membrane-bound organellar fraction. Bax and cytochrome c redistribution during apoptosis mainly takes place between the cytosol and mitochondria, thus immunoblot analysis of the organellar fraction is expected to reveal mitochondrial content of the molecules.

Immunoblot analysis. Electrophoresis and immunoblot analysis of proteins were performed in a NuPAGE Gel System. After being blocked with 2% BSA, the blots were incubated with specific primary antibodies overnight at 4°C. The blots were then exposed to the horseradish peroxidase-conjugated secondary antibody, and antigens on the blots were revealed using the enhanced chemiluminescence (ECL) kit (Pierce).

In vitro reconstitution of caspase activation. Reconstitution of caspase activation by adding exogenous cytochrome c to isolated cytosol was conducted as described in our previous work (13, 14, 16). Briefly, cell cytosol was extracted with 0.05% digitonin and concentrated to 4–5 mg protein/ml with 3-kDa cutoff microconcentrators. For reconstitution, 1 μl of 0.5 mg/ml cytochrome c, 1 μl of 10 mM dATP, and 0.5 μl of 200 mM MgCl2 were added to 7.5 μl of cell cytosol containing 25 μg protein. After 1 h of incubation at 30°C, the reconstitution mixture was transferred to caspase assay buffer containing DEVD.AFC to determine caspase activity.

Analysis of Bax oligomerization. DSP, a homobifunctional amine-reactive cross-linker, was utilized to analyze Bax oligomerization, as described in our previous work (14, 27). Briefly, cells were cross-linked with 1 mM DSP in phosphate-buffered saline for 30 min at room temperature under constant mixing. The cells were then fractionated with 0.05% digitonin to collect membrane fraction. Finally, the membrane fraction was subjected to electrophoresis and immunoblot analysis under nonreducing conditions.

Coimmunoprecipitation of caspase-9 and Apaf-1. Coimmunoprecipitation was conducted by a method modified from our previous work (14, 41, 47). Briefly, cell lysates were precleared by incubation with 1 μg of normal mouse serum and 30 μl of protein A/G agarose (Santa Cruz Biotechnology). The precleared samples were subsequently incubated for 2 h with 1 μg of caspase-9 immunoprecipitation antibody and 30 μl of protein A/G agarose. Immunoprecipitates were collected by centrifugation and dissolved in 2% SDS sample buffer for immunoblot analysis of caspase-9 and Apaf-1.

Statistics. Data were expressed as means ± SD (n ≥ 3). Statistical differences between various groups were determined by multiple comparisons, which were conducted by Tukey’s posttests following
ANOVA using the GraphPad Prism software. $P < 0.05$ was considered significantly different.

RESULTS

Suppression of ATP depletion-induced apoptosis by acidic pH. ATP depletion is a primary cause of ischemic renal cell injury in vivo and has been used in in vitro models for mechanistic studies (26). Our recent work examined the apoptotic mechanisms that are activated by ATP depletion in cultured renal tubular cells (13–15, 27, 31, 43). Using this well-characterized apoptotic model, we examined the effects of acidic pH in the current study. RPTC, a rat proximal tubular cell line, was subjected to ATP depletion by incubation with azide in glucose free medium. Subsequently, the cells were returned to full culture medium for recovery. As shown in Fig. 1A, significant amounts of cells developed apoptotic morphology following the treatment, showing cellular shrinkage and the formation of apoptotic bodies. Hoechst33342 staining further revealed nuclear condensation and fragmentation in these cells. Importantly, when the pH of the ATP depletion medium was decreased from 7.4 to 6.5, apoptosis was drastically reduced. For quantification, we counted the cells that showed typical apoptotic morphology (Fig. 1B). The basal level of apoptosis in control RPTC cells was $\sim 2\%$. After 3 h of ATP depletion at pH 7.4 and 1 h of recovery, 43% of cells became apoptotic. The percentage of apoptosis was slightly lower, when the pH of ATP depletion buffer was decreased to 7.0. However, a further decrease in pH to 6.5 or 6.0 induced a drastic reduction in apoptosis. Under these conditions, the rates of apoptosis were below 10% (Fig. 1B).

Inhibition of caspase activation during ATP depletion by acidic pH. To identify the apoptotic events that were blocked by acidic pH, we first examined caspase activation. The results are shown in Fig. 2. By enzymatic assays, we detected significant caspase activation during ATP depletion at pH 7.4 and 7.0. When the pH of ATP depletion buffer was lowered to 6.5 or 6.0, caspase activation was attenuated (Fig. 2A). The inhibitory effects of acidic pH were further indicated by immunoblot analysis of lamin B, an endogenous caspase substrate. As shown in Fig. 2B, lamin B of 72 kDa was cleaved into a characteristic fragment of 46 kDa following ATP depletion at pH 7.0 and 7.4 (lanes 4 and 5), but not at pH 6.0 or 6.5 (lanes 2 and 3). We also examined caspase activation in situ in intact cells by immunofluorescence of active caspase-3. As shown in Fig. 2C, many cells displayed immunofluorescence of active caspase-3 following ATP depletion at pH 7.4, while signifi-
cantly fewer positive cells were shown in the group that experienced ATP depletion at pH 6.5. Together, the results suggest that acidic pH suppressed caspase activation and apoptosis following ATP depletion in renal tubular cells.

Effects of acidic pH on cytochrome c during ATP depletion. Previous work by this and other laboratories demonstrated a role for the intrinsic mitochondrial pathway in tubular cell apoptosis following ATP depletion (12–14, 25, 31). A critical event of this pathway is the release of cytochrome c from mitochondria into cytosol. Thus we determined whether acidic pH inhibited cytochrome c release during ATP depletion. We initially analyzed cytochrome c release by immunoblotting following cellular fractionation. The results are shown in Fig. 3A. As expected, cytochrome c in control cells was detected in the mitochondrial fraction (lane 1). After ATP depletion, cytochrome c was lost from the mitochondrial fraction and appeared in the cytosolic fraction, indicating its release. Importantly, regardless of the pH variations from 7.4 to 6.0, cytochrome c release persisted and was comparable (lanes 2-5). The release of cytochrome c was confirmed by immunofluorescence analysis (Fig. 3B). Clearly, ATP depletion with azide induced cytochrome c release in many cells, resulting in a diffuse cytosolic staining. We subsequently counted the cells that released cytochrome c in representative fields. Consistent with the immunoblot results, no significant differences were shown between the groups at various pH levels (Fig. 3C). Together, the results suggest that acidic pH blocked apoptosis and caspase activation without affecting mitochondrial release of cytochrome c.

It was noticed that cytochrome c staining in some azide-treated cells was weaker than that of control cells (Fig. 3B). Interestingly, recent work by Zager et al. (48) showed that, in isolated mouse proximal tubules, cytochrome c after mitochondrial release traversed plasma membranes into the extracellular space. To determine whether cytochrome c was lost from azide-treated cells, we examined and compared cytochrome c in control and azide-treated cells by immunoblot. Cells were incubated for 3 h in glucose-free buffer in the absence or presence of azide. Whole cell lysates were then collected from these two groups of cells for immunoblot analysis. The results of samples collected from three separate experiments are shown in Fig. 3D (top blot). Densitometry of the blots showed that cytochrome c in the azide-treated group was 14.8 ± 10.2% lower than in the control group; however, the difference was not statistically significant (P > 0.05). We also collected and concentrated the incubation medium for cytochrome c immunoblot and did not detect significant signal (Fig. 3D, bottom blot). Apparently, cytochrome c leakage from azide-treated cells was not as extensive as that shown in other experimental models. The lower immunostaining signal was likely caused by...
the diffusion/dilution of cytochrome c within the cells after being released from mitochondria. In addition, some of the cells might be out of focus during microscopic recording.

Effects of acidic pH on Bax activation during ATP depletion. Our previous work suggested a critical role for Bax in mitochondrial disruption and cytochrome c release during ATP depletion of renal tubular cells (14–16, 27, 31). Bax was activated in ATP-depleted cells and translocated to mitochondria, where it oligomerized and presumably formed pathological pores on the outer membrane (14–16, 27, 31). Thus to further identify the apoptotic events that were blocked by acidic pH, we analyzed Bax activation. We initially examined

Fig. 3. Effects of acidic pH on cytochrome c (Cyt. c) release during ATP depletion. RPTC were incubated with 10 mM azide for 3 h in glucose-free buffer at pH 7.4, 7.0, 6.5, or 6.0. A: immunoblot analysis of cytochrome c. At the end of azide treatment, cells were fractionated into cytosolic and membrane-bound mitochondrial fractions for immunoblot analysis. This is a representative blot of 3 separate experiments. B: immunofluorescence of cytochrome c. Cells without (control) or with azide treatment at pH 6.5 were fixed and processed for immunofluorescence of cytochrome c. C: quantification of cells that released cytochrome c during ATP depletion. Images of cytochrome c immunofluorescence were analyzed to estimate the percentage of cells that released cytochrome c in cytosol. Data are expressed as means ± SD (n = 4). Multiple comparisons were conducted by Tukey’s posttests following ANOVA; no significant differences were shown between the acidic pH groups and the pH 7.4 group. D: cytochrome c leakage from cells during azide treatment. Cells were incubated for 3 h in glucose-free buffer (pH 7.4) in the absence or presence of azide. Whole cell lysates were then collected from these 2 groups of cells for immunoblot analysis (top blot). Incubation medium was also collected and concentrated with 3-kDa cutoff Microcons (Millipore) for cytochrome c immunoblot (bottom blot). Together, the results show that cytochrome c release during ATP depletion was not suppressed by acidic pH.
Bax translocation by immunoblotting following cellular fractionation. As shown in Fig. 4A, in control cells, the majority of Bax was detected in the cytosolic fraction (lanes 1). After 3 h of ATP depletion, significant amounts of Bax moved to the membrane-bound fraction enriched with mitochondria (lanes 2-5). Of note, Bax accumulation in the membrane fraction was similar at various pH levels. Bax translocation to mitochondria during ATP depletion was confirmed by immunofluorescence (Fig. 4B). Particularly, there was a population of cells that had intense Bax signal in mitochondria, showing a perinuclear staining. To quantify the cells with intense mitochondrial Bax, we counted these cells in representative fields; there was no difference between the groups that were ATP depleted at various pH levels (Fig. 4C). We further analyzed the oligomerization status of Bax (Fig. 4D). To preserve Bax oligomers, cells were subjected to chemical cross-linking by DSP. Cross-linked samples were then analyzed by immunoblotting. Clearly, Bax oligomerized in mitochondrial membranes, irrespective of the pH values during ATP depletion (Fig. 4D: lanes 1, 3, 5, 7). Of note, treatment of the cross-linked samples with DTT led to the dissociation of Bax oligomers (lanes 2, 4, 6, 8). This was due to the cleavage of the disulfide bond in DSP by the reducing agent. These results, together with the cytochrome c data, indicated that apoptotic events at the mitochondrial level were not abrogated by acidic pH.

Acidic pH blocks cytochrome c-stimulated caspase activation in isolated cytosol. Our analyses of Bax and cytochrome c suggest that acidic pH blocked apoptosis at the postmitochondrial level, downstream of cytochrome c release. Thus, to further identify the pH-sensitive event(s), we set up an in vitro system by adding exogenous cytochrome c to isolated cytosol. In this set of experiments, cytosol was isolated from control cells without ATP depletion and was free of cytochrome c (not shown). Exogenous cytochrome c was then added to the cytosol at various pH levels to induce caspase activation. As shown in Fig. 5A, cytochrome c stimulated caspase activation in isolated cytosol at pH 7.0 and 7.4, but not at acidic pH 6.0 or 6.5 (bar graph). Importantly, caspase-9 was processed into active forms following cytochrome c stimulation at pH 7.0 and 7.4 (blot: lanes 6 and 8), while the processing was markedly suppressed by lower pH (blot: lanes 2 and 4). The results were confirmed by using cytosol that was isolated from HK-2 cells, a human tubular cell line. As shown in Fig. 5B, both caspase activation and caspase-9 processing following cytochrome c stimulation were inhibited by acidic pH.

Acidic pH inhibits caspase-9 activation in apoptosome without blocking Apaf-1/caspase-9 association. In the intrinsic pathway of apoptosis, an immediate step following cytochrome c release is the binding of cytochrome c to Apaf-1, leading to the exposure of the CARD domain and the recruitment of caspase-9 to form a cytochrome c-Apaf-1-caspase-9 complex called apoptosome. Subsequently, caspase-9 is activated in apoptosome by a mechanism of proximity (7). Because cytochrome c-induced caspase-9 activation in isolated cytosol was inhibited by acidic pH (Fig. 5), we hypothesized that acidic pH might prevent the conformational changes in Apaf-1 that were needed for the recruitment and association of caspase-9. Recent work screened a panel of antibodies and identified a monoclonal antibody of human caspase-9 that is suitable for immunoprecipitation (40). Thus we determined the effects of buffer pH on the caspase-9/Apaf-1 association by immunoprecipitation (Fig. 6). Cytosol was isolated from HK2 cells and incubated at pH 7.4 or 6.5 in the absence or presence of cytochrome c. At the end of incubation, a portion of the samples was directly analyzed for caspase-9 and Apaf-1 by immunoblotting (lanes 1-4). The other portion was subjected to immunoprecipitation with the caspase-9-specific monoclonal antibody. Immunoprecipitates were then analyzed for caspase-9 and Apaf-1 by immunoblotting (lanes 5-8). As shown in Fig. 6, lanes 1-4, comparable amounts of caspase-9 and Apaf-1 were detected under various experimental conditions before immunoprecipitation. Consistent with previous observations, cytochrome c induced the processing of caspase-9 into its active fragments at pH 7.4 (lane 2) but not at pH 6.5 (lane 4). Following immunoprecipitation, significant amounts of caspase-9 were precipitated, including the intact and processed forms (lanes 5-8: bottom). In the absence of cytochrome c, limited amounts of Apaf-1 were coprecipitated along with caspase-9 (lanes 5 and 7: top). After cytochrome c stimulation, the coprecipitation or association between Apaf-1 and caspase-9 was markedly induced (lanes 6 and 8: top). Importantly, the association was not prevented by lowering the pH from 7.4 (lane 6) to 6.5 (lane 8). By densitometry of blots from four separate experiments, the amount of Apaf-1 that bound caspase-9/cytochrome c under pH 6.5 was similar to that bound under pH 7.4 (107 ± 36, if the signal of the pH 7.4 samples was arbitrarily set as 100). Collectively, the results indicate that acidic pH did not inhibit the recruitment of caspase-9 by Apaf-1 to form apoptosome; however, caspase-9 activation in this protein complex was suppressed.

DISCUSSION

Acidosis is a common feature of tissue ischemia (5, 24, 36). Under ischemia, cells within the affected tissues are forced to anaerobic glycolysis due to oxygen deprivation, leading to the accumulation of metabolic byproducts such as lactic acid, resulting in significant decreases in cellular pH. Acidosis is associated with ischemia of the brain, heart, liver, kidneys, and other organs (5, 24, 36). For example, in the brain, pH falls to 6.5 or lower following severe ischemic injury (36). Despite the recognition of acidosis in ischemic tissues, whether it is injurious or protective has been quite controversial. Acidosis is proposed to be a key detrimental factor for ischemic tissue damage (36). In addition to many earlier studies, the latest work by Xiong et al. (46) provided strong support for an injurious role of acidic pH. They showed that cells and animals lacking the acid-activated Ca^{2+}-permeable channel became resistant to ischemic injury in the brain (46). On the other hand, cytoprotective effects of mild acidosis have been demonstrated in vitro in different types of cells (5, 24). For example, in freshly isolated renal tubules, hypoxic injury was prevented, when the pH of the incubation buffer was decreased from 7.4 to 6.9, although the underlying mechanism was elusive (8). The current study specifically examined the effects of acidic pH on ATP depletion-induced tubular cell apoptosis, a process implicated in ischemic renal injury.

Our results show that tubular cell apoptosis as well as caspase activation were inhibited by acidic pH. Drastic inhibitory effects were shown, when the buffer pH was reduced from 7.0 to 6.5, suggesting a breakpoint within this range. Analysis of cytochrome c and Bax indicates clearly that up-
Fig. 4. Effects of acidic pH on Bax activation during ATP depletion. RPTC cells were incubated with 10 mM azide for 3 h in glucose-free buffer at pH 7.4, 7.0, 6.5, or 6.0. A: immunoblot analysis of Bax. At the end of azide treatment, cells were fractionated into cytosolic and membrane-bound mitochondrial fractions for immunoblot analysis of Bax. This is a representative blot of 3 separate experiments. B: immunofluorescence of Bax. Cells without (control) or with azide treatment at pH 6.5 were fixed and processed for immunofluorescence of Bax. C: quantification of cells that showed intense Bax signal in mitochondria. Images of Bax immunofluorescence were analyzed to estimate the percentage of cells with intense Bax signal in mitochondria. Data are expressed as means ± SD (n = 4). Multiple comparisons were conducted by Tukey’s posttests following ANOVA; no significant differences were shown between the acidic pH groups and the pH 7.4 group. D: Bax oligomerization. After azide treatment, cells were cross-linked with DSP. Mitochondrial fraction was then collected for immunoblot analysis under nonreducing (−DTT) or reducing (+DTT) conditions. Together, the results show that Bax activation during ATP depletion was not suppressed by acidic pH.
stream apoptotic events at the mitochondrial level were not suppressed by acidic pH. To identify the apoptotic events that were acidosis sensitive, we used an in vitro reconstitution system. When exogenous cytochrome \( c \) was added to isolated cell cytosol, caspase activation and caspase-9 processing were induced. Importantly, both events were attenuated by acidic pH. In the in vitro system, caspase-9 was expected to be the initiator caspase, which on activation would further process and activate downstream caspases. Thus, our results suggest that critical steps responsible for caspase-9 activation within the apoptosome were blocked by acidic pH. Nevertheless, cytochrome \( c \)-induced recruitment and association of caspase-9 by Apaf-1 were not inhibited by acidic pH. Taken together, it is concluded that acidic pH inhibited tubular cell apoptosis following ATP depletion by blocking caspase-9 activation in apoptosome.

Although the pH of the incubation buffer was vigorously controlled and monitored, we did not measure the pH within the cells. Nevertheless, the specific feature of the experimental model might facilitate a quick equilibration of protons across the plasma membrane. In these experiments, cells were depleted of ATP within 30 min of azide incubation in glucose-free medium (data not shown). Without ATP, the cellular capacity of maintaining ion homeostasis via channels and pumps was expected to be compromised. As a result, passive diffusion and transport of protons were mainly driven by concentration gradients, leading to the equilibration of intracellular pH with extracellular space. Importantly, the inhibitory effects of acidic pH on apoptosis and caspase activation in intact cells were also reproduced in the in vitro reconstitution system, where the pH of isolated cytosol was directly monitored.

It is noteworthy that acidic pH does not prevent or slow down ATP depletion under various experimental conditions (24). Similar results were shown in our experiments (not shown). Such a conclusion is also supported by the observation that upstream apoptotic events at the mitochondrial level were not suppressed by acidic pH.

Alterations of cellular pH have been shown to be involved in apoptosis. In T-lymphocytes, withdrawal of IL-7 led to Bax activation and consequent apoptosis, which were preceded by a rise in intracellular pH (22). Moreover, the active conformation of Bax could be induced by pH of 7.8 or higher (22). More recently, Tafani et al. (38) demonstrated changes in intracellular pH of HeLa cells in different apoptotic models. Although staurosporine treatment induced a rise in pH, TNF induced a decrease in cellular pH. Interestingly, prevention of pH alterations in either direction suppressed Bax activation and apoptosis in both models (38). These results support an important role for cellular pH in the initiation and progression of apop-
tosis. However, in our experimental model of ATP depletion, Bax activation and consequent cytochrome c release do not seem to be pH dependent. First, as discussed above, in ATP-depleted cells, pH is expected to equilibrate with the extracellular space and variations would be very limited. Under this condition, Bax activation and cytochrome c release were shown under a physiological pH of 7–7.4. Second, when pH was decreased to 6.5 and 6, neither cytochrome c release nor Bax activation was affected. In these experiments, we analyzed Bax and cytochrome c by several approaches including immunoblotting and immunofluorescence and obtained consistent results. Thus we conclude that pH alterations may not be the primary cause of mitochondrial events of apoptosis during ATP depletion, including Bax activation and cytochrome c release. These observations, however, do not exclude a role of intracellular pH in Bax activation and cytochrome c release in other apoptotic models, as shown previously (22, 38).

The fact that acidic pH inhibited apoptosis without blocking mitochondrial events suggests that the key pH-sensitive step is in the cytosol, downstream of cytochrome c release. This scenario is supported by the in vitro reconstitution experiments, showing that exogenous cytochrome c-stimulated caspase activation in isolated cell cytosol was suppressed, when the reconstitution pH was lowered from 7–7.4 to 6–6.5. Importantly, the processing of caspase-9, the initiator caspase in this system, was inhibited by lower pH, suggesting that a critical step blocked by acidic pH might be the activation of caspase-9 rather than downstream executioner caspases. In line with these observations, purified recombinant caspases including caspase-3 maintained significant enzymatic activity within a relatively broad pH range (37).

In the in vitro reconstitution system, cytochrome c after being added to the cytosol is expected to bind the WD40 domain of Apaf-1, leading to conformational changes of the latter and the exposure of the CARD domain. An exposed CARD domain in Apaf-1 recruits caspase-9 to form a protein complex called apoptosome. Caspase-9 is activated in apoptosis probably by proximity-induced autocalytosis (7). Apparently, a critical step for caspase-9 activation is the recruitment and association of caspase-9 by the adaptor protein Apaf-1, which holds caspase-9 molecules together to reach a concentration that is required for their autocatalytic activation (7). Thus, to explain the inhibitory effects of acidic pH in our experimental models, we initially hypothesized that lower pH might affect the recruitment and association of caspase-9 by Apaf-1 on cytochrome c stimulation. However, this possibility was not supported by our subsequent experiments of coimmunoprecipitation. It is clear that cytochrome c-induced caspase-9/Apaf-1 association was not prevented by pH 6–6.5. Thus, under mild acidic pH, cytochrome c after being released into the cytosol was able to induce conformational changes in Apaf-1 to recruit caspase-9 and form apoptosome; however, the apoptosome is not functional and caspase-9 is not activated under these conditions.

It remains unclear why the apoptosome formed under mild acidic pH does not function. Recently, well-controlled experiments by Beem et al. (4) investigated the mechanism of apoptosome formation using an in vitro system that was similar to that of our study. Their results led to a model of apoptosome maturation, starting with an initial 700-kDa complex, then dimerized into a 1,400-kDa intermediate, and finally transformed into a functional 700-kDa apoptosome. In this interesting model, caspase-9 is activated in the 1,400-kDa intermediate complex, while executioner caspases such as caspase-3 is proposed to be activated in the final 700-kDa complex. This model is supported by the observation that 150 mM KCl blocked the dimerization of the initial 700-kDa complex into the 1,400-kDa intermediate, while alkaline pH prevented the transformation of the 1,400-kDa intermediate into the final 700-kDa apoptosome (4). Should this occur in our system, it would not be far-fetched to speculate that acidic pH may inhibit the first step of apoptosome maturation, i.e., dimerization of the initial 700-kDa complex. Thus further investigations need to analyze the effects of acidic pH on the formation of various forms of apoptosome complexes.

In conclusion, this study examined the effects of acidic pH on ATP depletion-induced apoptosis in renal tubular cells. While apoptosis and caspase activation were suppressed by acidic pH, apoptotic events at the mitochondrial level including Bax activation and cytochrome c release were not affected. A critical step that was blocked by acidic pH was shown to be caspase-9 activation in apoptosome. These observations may have implications in apoptotic regulation during ischemic renal injury, a condition associated with acidosis.

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