Renal tubular epithelial cell apoptosis is associated with caspase cleavage of the NHE1 Na\(^{+}/H\(^{+}\) exchanger

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Departments of 1Medicine and 4Physiology and Biophysics, and 2Rammelkamp Center for Education and Research, Case Western Reserve University School of Medicine, Cleveland 44109; and 3Louis B. Stokes Veterans Administration Medical Center, Cleveland, Ohio 44106

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Renal tubular epithelial cell apoptosis is associated with caspase cleavage of the NHE1 Na\(^{+}/H\(^{+}\) exchanger. Am J Physiol Renal Physiol 284: F829–F839, 2003. First published November 26, 2002; 10.1152/ajprenal.00314.2002.—Renal tubular epithelial cell (RTC) apoptosis causes tubular atrophy, a hallmark of renal disease progression. Apoptosis is generally characterized by reduced cell volume and cytosolic pH, but epithelial cells are relatively resistant to shrinkage due to regulatory volume increase, which is mediated by Na\(^{+}/H\(^{+}\) exchanger (NHE1). We investigated whether RTC apoptosis requires caspase cleavage of NHE1. Staurosporine- and hypertonic NaCl-induced RTC apoptosis was associated with cell shrinkage and diminished cytosolic pH, and apoptosis was potentiated by amiloride analogs, suggesting NHE1 activity opposes apoptosis. NHE1-deficient fibroblasts demonstrated increased susceptibility to apoptosis, which was reversed by NHE1 reconstitution. NHE1 expression was markedly decreased in RTC apoptosis due to shrinkage due to regulatory volume increase, which is mediated by Na\(^{+}/H\(^{+}\) exchanger (NHE1). We investigated whether RTC apoptosis requires caspase cleavage of NHE1. Staurosporine- and hypertonic NaCl-induced RTC apoptosis was associated with cell shrinkage and diminished cytosolic pH, and apoptosis was potentiated by amiloride analogs, suggesting NHE1 activity opposes apoptosis. NHE1-deficient fibroblasts demonstrated increased susceptibility to apoptosis, which was reversed by NHE1 reconstitution. NHE1 expression was markedly decreased in apoptotic RTC due to degradation, and preincubation with peptide caspase antagonists restored NHE1 expression, indicating that NHE1 is degraded by caspases. Recombinant caspase-3 cleaved the in vitro-translated NHE1 cytoplasmic domain into five distinct peptides, identical in molecular weight to NHE1 degradation products derived from staurosporine-stimulated RTC lysates. In vivo, NHE1 loss-of-function C57BL/6SJL-squeaky mouse with adriamycin-induced nephropathy demonstrated increased RTC apoptosis compared with adriamycin-treated wild-type controls, thereby implicating NHE1 inactivation as a potential mechanism of tubular atrophy. We conclude that NHE1 activity is critical for RTC survival after injury and that caspase cleavage of RTC NHE1 may promote apoptosis and tubular atrophy by preventing compensatory intracellular volume and pH regulation.

TUBULAR ATROPHY IS A HALLMARK of chronic renal diseases and is superior to glomerular pathology as a histological predictor of clinical outcomes (41). We have previ-ously shown that renal tubular epithelial cell (RTC) apoptosis is a mechanism of tubular atrophy (20, 43). In the original descriptions of apoptosis morphology, Wyllie et al. (51) termed the process “shrinkage necrosis” due to reductions in cell volume observed during apoptosis. Apoptotic cell shrinkage is achieved by net loss of intracellular osmoles and H\(_2\)O, as well as by caspase-dependent proteolysis of housekeeping and structural proteins, which mediates cell disassembly. Many studies have also demonstrated that apoptosis is associated with a decrease in cytosolic pH (16, 27, 28, 30, 36, 46), which is required for activation of the caspase cascade (30, 45).

In contrast to neuronal cells and lymphocytes, epithelium-derived cells are relatively resistant to apoptosis following exposure to hypertonic extracellular conditions (6, 34), due to an enhanced capacity to rapidly expand intracellular volume through regulatory volume increase (RVI) pathways (17, 26, 31, 33). RVI is achieved by activation of the Na\(^{+}/H\(^{+}\) exchanger isoform NHE1 and, depending on the cell type, the anion exchanger (AE) 2 isoform of the Cl\(^{-}/HCO\(_3\)\(^{-}\) exchanger and/or the Na\(^{+}/K\(^{+}/2Cl\(^{-}\) symporter (26, 31, 33). The net effect is ion and H\(_2\)O influx, which leads to intracellular volume re-expansion. If RVI-dependent transporters are robustly activated after initiation of an apoptotic stimulus, restoration of intracellular volume may preemt apoptosis (26, 33). Alternatively, for a cell to undergo apoptosis, RVI must be overcome or inhibited (26, 33). Neither AE2 nor the types 1 or 2 bumetanide-sensitive cotransporter (BSC-1 or BSC-2, respectively) isoforms of the Na\(^{+}/K\(^{+}/2Cl\(^{-}\) symporter are expressed in proximal tubule (1, 15, 18), the nephron segment that demonstrates the most abundant apoptosis in animal models of progressive renal disease (20, 43). However, NHE1 is ubiquitously expressed, including within the proximal tubule, suggesting that NHE1 may be critical to RTC survival by promoting resistance to apoptotic cell shrinkage.

In addition to regulating cell volume via RVI, NHE1 mediates other housekeeping functions, such as intra-

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cellular pH (pH) regulation through electroneutral Na\(^+\) influx and H\(^+\) efflux. NHE1-dependent Na\(^+\)/H\(^+\) exchange has been linked to essential cell functions, such as proliferation (4, 32), whereas diminished NHE1 activity has been associated with lymphocyte apoptosis (27, 40). Moreover, NHE1 has recently been recognized to function as a scaffold for binding with ezrin, radixin, and moesin (ERM) (14), adapter molecules that link cytoskeleton to plasma membrane proteins, suggesting that NHE1 may facilitate apoptosis resistance by preserving cytoarchitecture and maintaining cell volume independent of Na\(^+\)/H\(^+\) antiporter functions.

Because predicted sequelae of NHE1 inhibition-cell shrinkage, intracellular acidification, and cytoskeleton collapse mimic the apoptotic phenotype, we investigated whether RTC apoptosis is regulated by NHE1 caspase cleavage. We find that RTC apoptosis is associated with caspase-dependent NHE1 degradation. Furthermore, cell culture and whole animal data demonstrate that NHE1 loss-of-function mutations render RTC susceptible to apoptosis. The data are consistent with a mechanism whereby NHE1 degradation causes RTC apoptosis and tubular atrophy, which prevents RVI and promotes intracellular acidosis, an optimum condition for caspase activity.

MATERIALS AND METHODS

Reagents. We used the following: amiloride, 4,6-diamidino-2-phenylindole (DAPI), ethyl-N-isopropylamiloride (EIPA), hexamethylenamiloride (HMA), staurosporine (STS), adriamycin hydrochloride (Sigma, St. Louis, MO); z-VAD-fmk, z-DEVD-fmk, Ac-DEVD-CHO (Calbiochem, La Jolla, CA); 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM (Molecular Probes, Eugene, OR); anti-poly-(ADP-ribose)polymerase (PARP) IgG, phycocerythrin (PE)-conjugated annexin V (Pharmingen, San Diego, CA); annexin V, anti-hemagglutinin (HA) IgG (Roche, Indianapolis, IN); horseradish peroxidase (HRP)-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA); green fluorescence protein (GFP) cDNA (Clontech, Palo Alto, CA); Red X-conjugated anti-mouse IgG, FITC-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA); \(^{35}\)S-methionine (ICN, Irvine, CA); C57BL/6;S.JL +/+ , C57BL/6;S.JL swe/swe +/, and C57BL/6;S.JL swe/swe mice (Jackson Laboratories, Bar Harbor, ME); COOH-terminal, HA epitope-tagged rat NHE1 cDNA (a gift from Dr. J. Orlowski, McGill University); and KR/A and E266I NHE1 mutant cDNAs (gifts from Dr. D. Barber, University of California at San Francisco). Rabbit polyclonal anti-NHE1 IgG was generated against an NHE1 cytoplasmic domain peptide as previously described (23) and affinity purified.

Cell lines. The human renal proximal tubule (HRPT) cell line was prepared by culture in Luria-Bertani-ampicillin. GFP, HA-NHE1, KR/A (inhibits ERM binding), and E226I (inhibits Na\(^+\)/H\(^+\) exchange) mutant NHE1 cDNAs were transiently transfected into cells according to previously described methods (21). Briefly, cells were plated in six-well dishes (0.25 \times 10\(^6\) cells/well) and cultured overnight in DMEM-F12 plus 10% fetal bovine serum to achieve 80% confluence. Cells were then washed and incubated with 100 \mu l of serum-free DMEM (Gibco-BRL) containing 6 \mu l of Fuge 6 transfection reagent (Roche) and 2.0–3.0 \mu g of plasmid DNA for 20 min at room temperature. Transfected cells were then cultured in complete media containing DMEM-F12 and 10% fetal bovine serum for an additional 24 h.

Immunoblot analysis. Methods have previously been described in detail (42). Whole cell lysates were prepared in boiling 2\times\) SDS sample buffer (125 mM Tris, pH 6.8, 2\% SDS, 5\% glycerol, 1\% β-mercaptoethanol, and 0.003% bromphenol blue). Samples were assayed for protein content using protein assay reagents (Bio-Rad, Hercules, CA). Proteins were denatured by boiling for 5 min, and samples (60 \mu l) were resolved by 8 or 14\% SDS-PAGE (Novex, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes, blocked with 5\% nonfat milk, and incubated with either anti-PARP (1:2,000, 1 h, room temperature) or anti-HA (1:5,000, 1 h, room temperature) antibodies, followed by HRP-conjugated secondary antibody (1:5,000, 1 h, room temperature). Band intensity was detected by enhanced chemiluminescence methods (Amersham Pharmacia Biotech, Arlington Heights, IL) and exposure to Kodak Biomax ML film. In some experiments individual bands were digitized by phosphorimager (Molecular Dynamics, Sunnyvale, CA), quantified with Image Quant 5 software (Molecular Dynamics), and normalized to control values.

Protein degradation by \(^{35}\)S-labeled pulse chase. Cells were cultured to subconfluence, washed with PBS, and incubated with \(^{35}\)S-methionine in methionine-free DMEM (0.1 \mu Ci/ml, 2 h, 37\°C). Cells were washed with PBS and cultured...
in complete media (0–6 h, 37°C) with or without STS and peptide caspase inhibitors. Protein lysates (200 μg per sample) were immunoprecipitated with anti-HA (1 μg) or anti-NHE1 IgG (1 μg) and resolved by SDS-PAGE according to previously described methods (42). Autoradiograms were developed from dried gels. In some experiments, individual bands were digitized by phosphorimager (Molecular Dynamics), quantified with Image Quant 5 software (Molecular Dynamics), and normalized to control values.

Immunocytochemistry and fluorescence microscopy. Methods have previously been described in detail (20, 21, 43). Cells were maintained on sterile glass coverslips within six-well plates, fixed in paraformaldehyde (4%, 10 min, room temperature), and blocked with 5% low-IgG BSA and 0.2% Triton X-100 (Sigma) for 30 min at room temperature. Cells were incubated with anti-HA IgG (1:200, 2 h, room temperature), followed by either red X-conjugated or FITC-conjugated antimouse IgG (1:200, 2 h, 4°C). Negative controls were cells incubated with isotype-identical IgG, which was immunoreactive with an irrelevant epitope. Coverslips were mounted in antifade, aqueous medium containing DAPI (Vectashield; Vector Laboratories) on standard microscope slides. Random photographs with a Spot Digital System camera and analyzed using Image Pro software.

Assay for caspase-3 cleavage of in vitro-translated NHE1. The DNA template for the DNA translation was created by PCR amplification of the NHE1 cytosolic domain (cNHE1) from rat cDNA with upstream primer 5'-CTACCGCTC-GAGCCCACTGCCCCAAGGACGTTCACTATGGCC-3' that contains Xho I restriction endonuclease, Kozak and ATG start sites, and downstream primer 5'-TGCTCTAGATGTG-CCTGCCCTTTGGGGATGAAGG-3' containing an Xba I restriction site and stop codon. The cNHE1 construct included the open reading frame encoding amino acids 447–820, which corresponds to the 58 COOH-terminal amino acids within the transmembrane domain and the entire cytosolic domain. The resulting DNA was digested with XhoI and XbaI (Gibco-BRL) and 1.1 kb product was cloned into pTNT vector (Promega, Madison, WI). PCR-generated cNHE1 nucleotide sequence was verified by automated sequencing (Cleveland Genomics, Cleveland, OH). [35S]Met-labeled cytoplasmic NHE1 substrate was generated using the reticulocyte lysate system (Promega) according to manufacturer’s instructions. Briefly, cNHE1 plasmid template (1 μg) was labeled with [35S]Met (50 μCi, 90 min, 37°C). Autoradiograms from dried gels yielded a single 45-kDa band within 2–3 h film exposure (not shown). [35S]Met-labeled, in vitro-translated cNHE1 (3 μl) was incubated with or without Ac-DEVD-CHO (100 μM, 2 h, 37°C), followed by 1–2 μl of purified caspase-3 (6 h, 30°C; Pharmingen) in 5 μl of caspase buffer (100 mM HEPES, pH 7.5, 10% sucrose, and 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate) according to published protocols (10, 22). Peptide products were resolved by 14% SDS-PAGE and examined for cleavage by autoradiography.

Animal models. C57BL/6 S. J. L. swe/swe mice harbor an NHE1 A1639T point mutation, which introduces a frameshift in the 11th and 12th NHE1 transmembrane domains and loss of NHE1-dependent Na+/H+ activity (13). Four-week-old swe/swe homozygotes have a brain phenotype that includes ataxia and seizures, but a gross renal phenotype was not observed, as determined by kidney histology and serum Na+, K+, Cl−, HCO3−, urea nitrogen, creatinine, and albumin concentrations. To identify the role of NHE1 in tubulointerstitial disease susceptibility, we induced nephropathy in 8-wk-old wild-type (C57BL/6 SJL +/-), C57BL/6.SJL swe/swe, and C57BL/6.SJL swe/swe mice by tail vein injection with adriamycin hydrochloride (10 μg/kg) (49). Mice with a C57BL/6 genetic background were specifically chosen because C57BL/6 SJL swe/swe mice survive to adulthood, in contrast to SJLJL swe/swe, which die at ~3 wk (3, 13). Furthermore, in contrast to BALB/c mice, C57BL/6 mice do not develop adriamycin nephropathy (49), allowing for more robust comparisons between potentially susceptible (swe/swe, swe/+ and resistant (+/+/+)) strains. Mice were killed 10 days after adriamycin infusion; swe/swe, swe/+ +, and +/+/+ animals were distinguished by immunoblotting liver lysates (20 μg protein/ lane) with anti-NHE1 antibodies (1:1,000). RTC apoptosis from frozen kidney sections was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays (Intergen, Purchase, NY) according to previously described methods (21, 43). Three sagittal sections from two mice per genotype were scanned for TUNEL-positive cells by two observers blinded to experimental conditions. Kidney area was determined with a Spot Digital System camera and Image Pro software, and data are expressed as apoptotic RTC/mm². The animal care protocol was approved by the Institutional Animal Care and Use Committee at Case Western Reserve University School of Medicine.

Statistics. Data are representative of three to five experiments per condition. Graphical results are expressed as means ± SE unless otherwise indicated. Comparisons between paired samples were made by the Student’s t-test. Comparisons between groups containing more than two samples were made by one-way analysis of variance with the Bonferroni, Student-Newman-Keuls, or Kruskal-Wallis tests as appropriate. Significance is defined as P < 0.05.

RESULTS

Apoptotic RTC are shrunken and acidic. To determine whether apoptotic RTC develop reduced cell volume and cytosolic pH, as has been described in leukocytes, we stimulated cultured RTC to undergo apoptosis with STS. Cell size and pH were determined by flow cytometry. As shown in Fig. 1, a greater proportion of RTC incubated with STS displayed smaller cell volumes and lower pH compared to nonapoptotic RTC. Relative cytosolic pH was measured by 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein -AM fluorescence (y-axis) and relative cell volumes by light scatter characteristics (x-axis). Results are representative of 5 separate experiments.

Fig. 1. Apoptotic renal tubular epithelial cells (RTC) are shrunken and acidic. Subconfluent monolayers of cultured RTC were incubated with or without staurosporine (STS, 1 μM, 5 h, 37°C). Apoptosis was determined by annexin V labeling (apoptotic cells are red, nonapoptotic cells are green). Relative cytosolic pH was measured by 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM fluorescence (y-axis) and relative cell volumes by light scatter characteristics (x-axis). Results are representative of 5 separate experiments.
cell volumes and lower cytosolic pH, similar to STS- and Fas-induced Jurkat T cell apoptosis (30). These data demonstrate that the apoptotic RTC phenotype includes shrinkage and acidification, consistent with a role for NHE1 inhibition.

**Hypertonicity stimulates RTC apoptosis.** Epithelial cells are relatively resistant to apoptosis induction by anisotonic conditions due to robust RVI (6, 34), which is mediated by multiple transporters (26, 31, 33). To determine whether cell stress imposed by hypertonicity causes apoptosis, we exposed RTC to increasing extracellular concentrations of the impermeant sugars mannitol and sucrose or NaCl and then simultaneously assayed them for apoptosis by annexin V labeling of externalized phosphatidylserine and chromatin condensation. Figure 2 shows that hypertonicity caused RTC apoptosis in response to all three stimuli in a concentration-dependent fashion, indicating that RVI was surmounted and RTC were susceptible to hypertonic stress-induced apoptosis.

**Hypertonicity stimulates RTC apoptosis by an NHE1-dependent mechanism.** To determine the role of NHE1 in apoptosis induced by hypertonic conditions, we examined the effect of NHE1 inhibitors on RTC apoptosis, assayed by DAPI labeling of condensed chromatin (Fig. 3A) and annexin V labeling of externalized phosphatidylserine (Fig. 3B). These studies show that the Na+/H+ inhibitor amiloride caused modest apoptosis but significantly potentiated RTC apoptosis from hypertonic stress. Because amiloride, particularly at high concentrations, may inhibit multiple sodium transporters, including other NHE isoforms expressed in proximal tubule, NaCl-induced apoptosis was assayed following preincubation with NHE1-specific amiloride analogs EIPA and HMA (35, 47). Figure 3, C and D, demonstrates that, like amiloride, EIPA and HMA caused modest apoptosis and both inhibitors enhanced hypertonic NaCl-dependent apoptosis (HMA > EIPA), suggesting that NHE1 inactivation potentiates RTC apoptosis induced by stresses, such as hypertonicity.

**Hypertonicity-induced RTC apoptosis is mediated by caspase-3 activation and decreased NHE1 expression.** To determine whether NHE1 is linked to caspase activation due to hypertonic stimuli, we incubated RTC with hypertonic NaCl and amiloride and then probed for activation of caspase-3, which is the final downstream executioner caspase in many apoptosis signaling cascades. Figure 4A shows that amiloride accentuated hypertonicity-induced cleavage of the caspase-3 substrate PARP, suggesting that NHE1 activity opposes apoptosis, perhaps by inhibiting decreases in cytosolic pH, which enhance caspase-3 activity (30, 45). Figure 4, B and C, demonstrates that hypertonic NaCl induction of caspase-3 activity was associated with diminished NHE1 expression, indicating that NHE1 may represent a caspase-3 target, which is consistent with the possibility that NHE1 dysfunction could contribute to the acidic and shrunken cell phenotype, as shown in Fig. 1.

**RTC apoptosis under isotonic conditions is associated with decreased NHE1 expression.** Because the data indicated that cell shrinkage-induced RTC apoptosis is mediated by NHE1 inhibition and caspase-3 activation, we queried whether NHE1 could be a caspase-3 substrate. To explore this possibility, we transiently cotransfected HEK 293 cells with an NHE1 cDNA construct containing a carboxy-terminal HA tag and GFP cDNA (to mark transfected cells), followed by
then maintained in hypertonic media (DMEM-F12 preincubated with amiloride at indicated concentrations for 2 h and then incubated to stimulate caspase-3-dependent apoptosis. Nuclear morphology and NHE1 expression patterns were determined by standard, fluorescence microscopy. Figure 5, A and B, demonstrates representative transfected, apoptotic, and nonapoptotic cells. Approximately 20% of all cells underwent apoptosis, but only a small percentage of transfected, apoptotic cells expressed NHE1 on the cell surface (Fig. 5, C and E). Conversely, almost all transfected, nonapoptotic cells expressed NHE1 in a plasma membrane distribution (Fig. 5, C and E). Similar results were observed in RTC (data not shown). The results from these experiments suggest that NHE1 is cleaved during apoptosis and that loss of NHE1 expression renders cells susceptible to apoptosis.

**NHE1 reconstitution promotes resistance to apoptosis.** To further investigate the role of NHE1 in apoptosis, we compared NHE1-deficient PS120 cells, which were derived from Chinese hamster ovary fibroblasts (37), and NHE1-expressing control fibroblasts (CCL39 cells) for susceptibility to STS-induced apoptosis. As shown by DAPI and annexin V assays in Fig. 6, A and B, respectively, apoptosis was observed in a significantly greater percentage of PS120 cells compared with the CCL39 cells, consistent with a recent report in these two cell lines (2). NHE1 function was subsequently addressed by add-back experiments, in which PS120 cells were transiently transfected with increasing concentrations of NHE1 then stimulated with STS and assayed for apoptosis. Figure 6C demonstrates that NHE1 reconstitution in PS120 cells conferred resistance to apoptosis. At the highest NHE1 expression levels, apoptosis was equivalent to control CCL39 cells (Fig. 6, A and B).

To assess which NHE1 domains are required for apoptosis resistance, we transiently transfected PS120 cells with wild-type NHE1, an NHE1 mutant that does not bind ERM proteins due to multiple K/A or R/A substitutions in residues 553–564 (KR/RA) or an Na+/K+ exchange-defective NHE1 construct containing a point mutation in the third cytoplasmic loop (E266I). Significant differences in transfection efficiency were not observed between groups (not shown). Cells were then stimulated with STS and assayed for apoptosis. Similar to results in Fig. 6, A and B, PS120 cells were susceptible to apoptosis, which was reversed by transfection of wild-type NHE1 expression (Fig. 6D).

**Fig. 4.** Hypertonicity-induced RTC apoptosis is mediated by caspase-3 activation and decreased NHE1 expression. A: RTC were preincubated with amiloride at indicated concentrations for 2 h and then maintained in hypertonic media (DMEM-F12 + 300 mM NaCl, 5 h, 37°C). Whole cell lysates were probed for poly(ADP-ribose)/polymerase (PARP) expression by immunoblot analysis. Caspase-3 activation was determined by PARP cleavage, which is indicated by the arrow. Human renal proximal tubule (HRPT) cells (B) or hemagglutinin (HA) epitope-tagged NHE1-transfected HEK 293 cells (C) were incubated in serum-free media (–) or in serum-free media plus 300 mM NaCl (+) for 6 h at 37°C. Whole cell lysates were immunoblotted with anti-NHE1 IgG (B, top) or anti-HA IgG (C, top). Lysates were blotted with anti-PARP IgG (B and C, bottom). The 85-kDa PARP cleavage product is marked by the arrow. Results are representative of 3 separate experiments. M, molecular weight.

**Fig. 5.** RTC apoptosis under isotonic conditions is associated with decreased NHE1 expression. HEK 293 cells were cotransfected with carboxy-terminal, HA-tagged NHE1 and green fluorescence protein (GFP) cDNAs (1 μg/well with each vector) then incubated with STS (1 μM, 5 h, 37°C) to induce apoptosis. Fluorescence (not confocal) micrographs show transfected cells (green, A), apoptosis (fragmented nuclei stained with DAPI (blue) (B), NHE1 expression by immunocytochemical staining with biotinylated anti-HA IgG and Texas red-conjugated streptavidin (red, C), and merged images (D) from A–C. Results are representative of 3 experiments. E: 200 GFP-positive cells per experiment were scored for apoptosis, as defined by DAPI labeling of condensed chromatin and plasma membrane NHE1 expression, by immunocytochemical staining, as described in C. Quantitation of NHE1 expression in apoptotic vs. nonapoptotic cells is shown in E. Results represent means ± SE from 3 experiments. *P < 0.05 compared with GFP-positive, apoptosis-negative group by Student’s t-test.
Expression of KR/A mutant NHE1 partially restored cell viability (Fig. 6D), implying that NHE1-ERM binding is not the sole determinant of apoptosis resistance. Importantly, E266I expression did not rescue cells from apoptosis (Fig. 6D), which indicates that Na⁺/H⁺ exchange is critical for apoptosis resistance.

**RTC apoptosis leads to diminished NHE1 expression by protein degradation.** Because NHE1 has a long (~24 h) half-life (9, 12) and is not significantly regulated by membrane cycling (38), rapid NHE1 disappearance with STS incubation suggests an NHE1 degradation mechanism, rather than suppressed synthesis. To determine more definitively whether STS-induced decreases in cell surface NHE1 were due to protein degradation, we conducted 35S-labeled pulse-chase experiments in HEK 293 cells transfected with HA-tagged NHE1 cDNA, metabolically labeled with [35S]methionine, and then treated with 1 μM STS. Immunoprecipitation with anti-HA IgG revealed diminished 35S-labeled NHE1 beginning 2.5 h after STS incubation, which was markedly more pronounced at 5–6 h (Fig. 7), indicating that STS stimulates NHE1 degradation. 35S-labeled NHE1 levels did not appreciably change in unstimulated cells from 0 to 4 h, consistent with the long NHE1 half-life (not shown). More importantly, 35S-labeled NHE1 levels were significantly greater in unstimulated compared with STS-stimulated cells at 4 h (not shown), further indicating that NHE1 is degraded with apoptosis. Because STS is a known caspase-3 activator, the data also suggest a caspase-3 mechanism of NHE1 degradation.

Although HA-tagged proteins have been successfully employed as caspase substrates (8, 11, 48), because the HA epitope sequence contains a putative caspase-3 cleavage site (YPYDPVDYA), pulse chase experiments were also conducted in untransfected RTC, followed by immunoprecipitation of endogenous NHE1 with rabbit polyclonal anti-human NHE1 IgG raised against the membrane-proximal cytoplasmic domain (23). These studies also revealed NHE1 degradation (Fig. 9A), with a similar kinetic pattern as in Fig. 7, demonstrating that NHE1 is degraded during apoptosis.
NHE1 is degraded by caspase-3. To determine whether apoptosis-associated NHE1 degradation is due to caspase cleavage, we transiently transfected RTC with carboxy-terminal HA, epitope-tagged NHE1, and stimulated them with STS to undergo apoptosis in the presence or absence of the cell-permeable, broad-spectrum peptide caspase inhibitor z-DEVD-fmk or the peptide caspase-3 inhibitor z-DEVD-fmk. Whole cell lysates were probed for NHE1 expression and PARP cleavage by immunoblot analysis. Figure 8 demonstrates that STS induced concomitant caspase-3 activity and NHE1 degradation in HEK 293 cells, and both caspase-3 activity and NHE1 degradation were partially inhibited by z-DEVD-fmk or z-VAD-fmk preincubation. Similar results were observed in RTC (Fig. 8B). These data strongly suggest that NHE1 is cleaved by caspase-3 during apoptosis.

To further define whether NHE1 is a caspase substrate, we metabolically labeled RTC, induced them with STS to undergo apoptosis, and analyzed anti-NHE1 immunoprecipitates for potential caspase cleavage products. Figure 9A reveals simultaneous NHE1 degradation and appearance of several bands ranging in size from $M_r$ 15 to 32 kDa, suggesting that NHE1 is cleaved by caspase-3. To directly assess whether NHE1 is a caspase target, we incubated in vitro-translated cNHE1 with recombinant caspase-3 in the presence and absence of the caspase-blocking peptide Ac-DEVD-CHO and then evaluated it for degradation. As shown in Fig. 9B, in vitro degradation of cNHE1 by caspase-3 resulted in the generation of peptides identical in $M_r$ compared with NHE1 peptides derived from STS-stimulated whole cell lysates (Fig. 9A) in the absence, but not presence, of the peptide caspase inhibitor. A 37-kDa band was observed in control conditions (Fig. 9B), which was not inhibited by Ac-DEVD-CHO, suggesting that this product was nonspecifically generated by caspase buffer alone. However, the other

![Fig. 8. NHE1 is degraded by caspases.](image)

![Fig. 9. A: [35S]methionine-labeled RTC, treated with 1 μM STS for indicated times, were immunoprecipitated with affinity-purified rabbit polyclonal anti-NHE1 IgG. B: [35S]methionine-labeled, in vitro-translated cNHE1 protein (3 μl) was preincubated with or without the peptide caspase inhibitor Ac-DEVD-CHO (DEVD, 100 μM, 2 h) and then with recombinant caspase-3 (casp-3, 1–2 μl, 6 h, 30°C) as described in MATERIALS AND METHODS. The reaction product was resolved by 14% SDS-PAGE, and the dried gel was exposed to film for 3 h. The most prominent, common bands from A and B are demarcated by arrows. Results are representative of 3 separate experiments. cNHE1, NHE1 cytosolic domain.)
DEVD-inhibitable bands (shown by arrows) represent true caspase cleavage products. Together, data from Figs. 8 and 9 provide compelling evidence that NHE1 is a caspase-3 substrate.

**NHE1 regulates RTC apoptosis in vivo.** To test the role of NHE1 inactivation as a mechanism of RTC deletion in an in vivo model of progressive renal disease, wild-type (C57BL/6.SJL +/+), NHE1 loss-of-function mutant (C57BL/6.SJL swe/swe), and heterozygote (C57BL/6.SJL swe/+ ) mice were genotyped by immunoblotting liver lysates (20), RTC apoptosis was rarely observed in wild-type controls (Fig. 10A), consistent with a failure of adriamycin to cause nephropathy in mice with a C57BL/6 genetic background (49). However, significant increases in RTC apoptosis were observed in C57BL/6 swe/swe and C57BL/6 swe/+ mice (Fig. 10, B and C). Because neither C57BL/6 mice infused with adriamycin nor NHE1 loss-of-function mutant C57BL/6 swe/swe mice demonstrate histological or functional kidney abnormalities (13, 49), the data suggest that NHE1 inhibition unmasked a renal phenotype in adriamycin-treated C57BL/6 mice. In accordance with our previous reports demonstrating that RTC apoptosis precedes and contributes to tubular atrophy (20), these studies indicate that NHE1 confers cytoprotection, whereas loss of RTC NHE1 function is associated with apoptosis and tubular atrophy.

**DISCUSSION**

NHE1 is ubiquitously expressed, and activation has been linked to vital housekeeping functions such as cell volume regulation (31, 33) and growth factor-dependent proliferation (4, 32, 44). Decreased NHE1 activity has been associated with lymphocyte apoptosis (27, 40), but a specific mechanism of NHE1 inhibition in apoptosis has not previously been described. A major finding in our study is that RTC NHE1 is inhibited by caspase cleavage. The results have potentially broad implications to disease pathogenesis, inasmuch as the data were generated in both epithelial and mesenchymal cells and in response to apoptosis induction by hypertonic stress or STS.

Many epithelium-derived cell lines were previously considered to be resistant to apoptotic cell volume reduction due to robust expression and function of RVI pathway components (6). However, our data demonstrate that RTC RVI can be overcome, permitting apoptosis to proceed. Of the transporters that mediate RVI, we focused on NHE1 partly because it is responsible for intracellular volume regulation, and an anticipated consequence of NHE1 inhibition, cell shrinkage, is a characteristic apoptotic feature. Indeed, we found that RTC apoptosis is associated with diminished cell volume, consistent with NHE1 inhibition. Our data do not exclude roles for regulatory volume decrease pathways, which can be activated in apoptosis (33). In addition, other transporters (7, 29, 52) may participate in RTC RVI triggered by apoptotic stimuli. For example, inhibition of NHE3, which is expressed on the apical proximal RTC membrane, could conceivably contribute to RTC shrinkage and acidosis. However, because hypertonic cell shrinkage suppresses NHE3 activity (19), we reasoned that apoptotic cleavage of NHE3 was unlikely to result in significant further suppression of transporter activity. Inhibition of other transporters that have been implicated in RVI, such as the AE2 Cl⁻/HCO₃⁻ exchanger or the BSC-1 and BSC-2
isoforms of the Na\(^{+}/K\(^{+}/2Cl\(^{-}\) cotransporter, could also be involved in RTC apoptosis, by impeding intracellular volume expansion. However, prominent functions for these proteins in proximal RTC apoptosis are unlikely, since none are abundantly expressed in the proximal tubule (1, 15, 18).

In addition to cell shrinkage, RTC NHE1 degradation was also associated with intracellular acidification (16). The data are in agreement with studies in leukocyte cell lines, which demonstrate that apoptosis is preceded by decreased pH (16, 27, 36). Importantly, NHE1 stimulation prevented intracellular acidification and abrogated apoptosis (16, 36), indicating NHE1 maintenance of cell volume and resistance to apoptosis. Be- and the cytoskeleton may be important for maintenance that interactions between other NHE1 domains bane of cell volume and resistance to apoptosis. Be-

Evidence to support the hypothesis that NHE1 is cleaved by caspases includes apoptosis-dependent loss of NHE1 expression due to protein degradation, rescue of NHE1 expression by preincubation with cell-permeable peptide caspase inhibitors, and direct cleavage of in vitro-translated NHE1 by caspase-3. Although consensus caspase cleavage sites are identified in the carboxy-terminal human NHE1 cytosolic tail (e.g., 755-DEED-758), deletion mutation studies predict that cleavage at this site would not result in Na\(^{+}/H\(^{+}\) exchange-dependent osmoregulatory dysfunction (5). Furthermore, cleavage at distal carboxy-terminal site(s) would result in generation of very small fragments and, therefore, would not account for the peptide band pattern observed in Fig. 9, indicating that cleavage at additional membrane-proximal, noncanonical sites is required. Further degradation of NHE1 and/or NHE1 caspase cleavage products by other protease pathways is also possible, although preliminary studies revealed that STS-induced changes in NHE1 expression are not altered by pretreatment with the proteosome inhibitors lactacystin, MG132, and PS-1 (Wu KL and Schelling JR, unpublished observations).

Although neither in vitro stimulus of apoptosis (STS, hypertonicity) is encountered by RTC in vivo, these agents were employed to mimic cell stresses that result in RTC apoptosis in vivo. We have previously shown that RTC apoptosis and tubular atrophy are caused by the in vivo stresses hypoxia and Fas activation in murine models of progressive renal disease (20, 43). In the current studies, the in vivo role of NHE1 was established by demonstration of increased RTC susceptibility to apoptosis in NHE1-deficient mice with Adriamycin-induced nephropathy. To maximize the likelihood of detecting apoptotic RTC before obliteration of tubulointerstitial architecture by renal scarring, we killed mice after only 10 days. Because of the short observation interval, animals did not develop tubulointerstitial pathology, although we predict that the natural history of enhanced RTC apoptosis is tubular atrophy and interstitial fibrosis.

NHE1 is commonly referred to as a housekeeping protein, implying that it is pedestrian and unregulated. To the contrary, NHE1 has been shown to mediate vital cell functions, and the current studies establish a new role for NHE1 as a defender against RTC death. We speculate that in initial stages of RTC apoptosis in vivo, e.g., due to inflammation or uremia, NHE1 is likely to be activated in response to cell volume reduction cues. NHE1-dependent RVI may then be sufficient to prevent further cell volume shrinkage and perhaps even promote cell survival, provided the apoptotic stimulus is not too robust. However, once NHE1 is cleaved by caspases, the combined sequelae of NHE1 inhibition, cytosolic acidification, and cell shrinkage promote inexorable RTC apoptosis by optimizing pH, for further caspase activity and by
bringing caspases in proximity to substrates via cell shrinkage.

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