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Dysfunction of regulatory volume increase is a key component of apoptosis

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Abstract **Sustained cell shrinkage is a major hallmark of apoptotic cell death. In apoptotic cells, whole cell volume reduction, called apoptotic volume decrease (AVD), proceeds until fragmentation of cells. Under non-apoptotic conditions, human epithelial HeLa cells exhibited a slow regulatory volume increase (RVI) after osmotic shrinkage induced by exposure to hypertonic solution. When AVD was induced by treatment with a Fas ligand, TNF-α or staurosporine, however, it was found that HeLa cells failed to undergo RVI. When RVI was inhibited by combined application of Na^+/H^+ exchanger (NHE) and anion exchanger blockers, hypertonic stress induced prolonged shrinkage followed by caspase-3 activation in HeLa cells. Hypertonicity also induced apoptosis in NHE1-deficient PS120 fibroblasts, which lack the RVI response. When RVI was restored by transfection of these cells with NHE1, hypertonicity-induced apoptosis was completely prevented. Thus, it is concluded that RVI dysfunction is indispensable for the persistence of AVD and induction of apoptosis.**

Keywords: Apoptosis; Apoptotic volume decrease; Regulatory volume increase; Na^+/H^+ exchanger

1. Introduction

Apoptosis is an essential event for embryogenesis, organ development, tissue homeostasis, somatic cell turnover, and immune system maturation [1–3], and also underlies pathogenesis of degenerative diseases, viral infections, cancer, AIDS and autoimmune disorders [4–6]. A major hallmark of apoptosis is normotonic cell shrinkage [7,8]. A reduction of whole cell volume called apoptotic volume decrease (AVD) [9,10] continues until cell fragmentation in cells undergoing apoptosis. The AVD induction precedes cytochrome *c* release, caspase-3 activation, DNA fragmentation, cell fragmentation and eventual cell death [9,10]. The AVD is indispensable for the apoptosis progression, because the succeeding apoptotic events were prevented when the AVD induction was inhibited [9,10].

Most types of normal cells regulate their volume after shrinkage by a process called regulatory volume increase (RVI) which is accomplished by NaCl uptake mediated by parallel operation of Na^+/H^+ exchanges (NHE) and Cl^-/HCO_3^- anion exchanges (AE), by operation of Na^+–K^+–$2Cl^-$ cotransporters (NKCC) or Na^+–Cl^- contransporters (NCC), and/or by shrinkage-induced activation of Na^+-permeable cation channels [11–14]. In apoptotic cells, however, persistent cell shrinkage takes place. Thus, the RVI mechanism must be either inhibited or overridden in apoptotic cells [15]. The first purpose of the present study was to examine whether apoptotic stimulation causes inhibition of RVI in human epithelial HeLa cells.

Bortner and Cidlowski [15] demonstrated that hypertonicity-induced shrinkage leads to apoptosis in lymphoid cells that lack the RVI response but not in several other RVI-exhibiting cells including HeLa cells. Their data suggest that not only the induction of AVD, but also the dysfunction of RVI, are key components of the apoptosis process. The second purpose of the present study was to examine whether restoration of the ability to undergo RVI rescues cells from hypertonicity-induced apoptosis.

2. Materials and methods

2.1. Cell culture and apoptosis stimulation

HeLa cells were cultured in 10% FBS/MEM at 37 °C in 5% CO_2/95% air under humidified conditions. Chinese hamster ovary PS120 fibroblasts and PS120/NHE1 cells that were stably transfected with cDNA encoding Na^+/H^+ exchanger isoform 1 (NHE1) were provided by Dr. S. Wakabayashi (National Cardiovascular Center Research Institute, Osaka, Japan) and cultured in 10% FBS/DMEM, as previously described [16].

To stimulate apoptosis, HeLa cells were treated with 4 μM staurosporine (STS), 500 ng/ml anti-Fas antibody, or 2 ng/ml tumor necrosis factor-α (TNF-α) plus 1 μg/ml cycloheximide (CHX).

2.2. Cell volume measurements

Cell volume was measured by an electronic sizing technique with a Coulter-type cell size analyzer (CDA-500; Sysmex, Kobe, Japan), as previously described [17]. The mean volume of the population was calculated by a computer from the cell volume distribution determined by comparison with latex beads of known volume. Isoosmotic (300 mosmol/kg-H_2O) solution was made of serum-free DMEM containing 20 mM $NaHCO_3$ and 20 mM HEPES/ NaOH (pH 7.4). Hypertonic solution (600 mosmol/kg-H_2O) was prepared by adding 300 mM mannitol or 150 mM NaCl to the isotonic solution.

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Abbreviations: AE, anion exchanger; AVD, apoptotic volume decrease; CHX, cycloheximide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; FasL, Fas ligand; NHE1, Na^+/H^+ exchanger isoform 1; RVI, regulatory volume increase; STS, staurosporine; TNF-α, tumor necrosis factor-α

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2.3. Cell viability assays

To estimate total cell viability, mitochondrial dehydrogenase activity was measured by a colorimetric MTT assay using the Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol, as previously described [9,18].

2.4. Apoptosis assays

Caspase-3 activity measurements and internucleosomal DNA fragmentation assays were performed, as previously described [9,18]. Briefly, caspase-3 activity was measured using the CaspACE Assay System (Promega, Madison, WI). To exclude the involvement of other related proteases, the difference in fluorescence when a specific inhibitor of caspase-3 was absent compared to when it was present was observed. For DNA ladder assays, cells cultured in 24-well plates were digested and incubated in mammalian cell lysis buffer (0.1% SDS, 10 mM EDTA, 10 mM Tris, pH 8.0) containing 20 μg/ml RNase (Nacalai Tesque, Kyoto, Japan) at 37 °C for 1 h and 0.5 mg/ml proteinase K (Nacalai Tesque). The chromosomal DNA was analyzed by agarose gel electrophoresis (2%) followed by staining with ethidium bromide.

2.5. Statistical analysis

The data, presented as means ± S.E.M., were statistically analyzed using the unpaired *t*-test or the Welch *t*-test when variances were heterogeneous. Differences were considered significant when P was <0.05.

3. Results

3.1. Impairment of RVI in apoptotic HeLa cells

When treated for 2 h with a death receptor-mediated apoptosis inducer, TNF-α (plus CHX) or Fas ligand (FasL), or with a mitochondrion-mediated apoptosis inducer, STS, HeLa cells exhibited AVD (data not shown), as previously observed [9,10]. Both control and HeLa cells exhibiting AVD responded to a hypertonic challenge with rapid shrinkage, as shown in Fig. 1. In AVD-exhibiting cells treated with TNF-α (Fig. 1A), FasL (Fig. 1B) or STS (Fig. 1C), osmotic shrinkage persisted without subsequent volume regulation (filled symbols), whereas control cells showed gradual volume recovery whether or not they received CHX treatment (open symbols). These data indicate that the RVI mechanisms are inhibited in HeLa cells that are stimulated with either a death receptor- or mitochondrion-mediated apoptosis inducer and are exhibiting AVD.

3.2. Hypertonicity-induced caspase-3 activation in HeLa cells treated with RVI inhibitors

Hypertonic stress alone was shown to lead to apoptosis in lymphoid cells lacking the RVI mechanism but not in other cell types that exhibit RVI, such as epithelial HeLa cells [15]. However, it is not clear whether osmotic shrinkage causes apoptosis in lymphoid cells only or whether it causes apoptosis in other cell types as well when their RVI is inhibited. Thus, we next examined the effect of RVI blockers on caspase-3 activity in HeLa cells under isotonic and hypertonic conditions.

In the presence of both an anion exchanger (AE) blocker, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, 200 μM), and an NHE blocker, amiloride (100 μM), it was found that RVI after exposure to hypertonic solution (which was prepared by adding 300 mM mannitol) was almost completely suppressed (Fig. 2A, filled circles). As shown in Fig. 2B, significant activation of caspase-3 was observed in the presence of both blockers after 6-h exposure to hypertonic solution (fourth column) but not isotonic solution (second column). In contrast, significant activation of caspase-3 was not induced by hypertonic stress in the absence of DIDS and amiloride (Fig. 2B, third column) in HeLa cells that could exhibit RVI under this condition (Fig. 2A, open squares). These data indicate that even non-lymphoid HeLa cells subjected to osmotic shrinkage undergo apoptosis when RVI is inhibited by DIDS and amiloride.

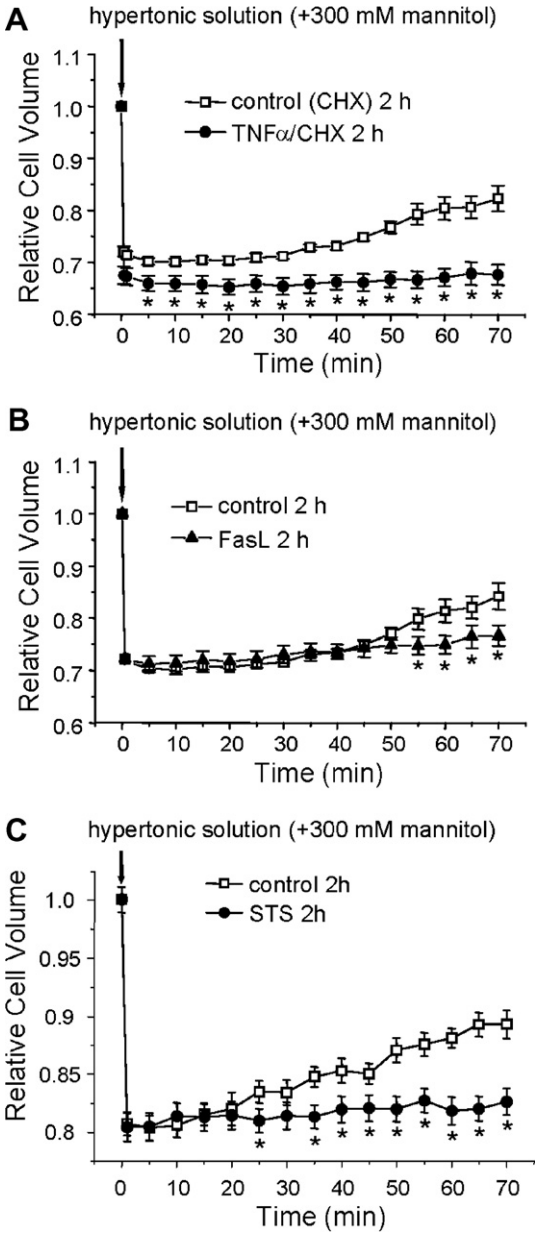

Fig. 1. Effects of 2-h exposure to apoptosis inducers on the RVI after osmotic cell shrinkage induced by a hypertonic challenge. Relative cell volume, normalized to the cell volume under isotonic conditions (300 mosmol/kg-H_2O), was plotted against time after application of hypertonic solution (600 mosmol/kg-H_2O with 300 mM mannitol). HeLa cells were pretreated with vehicle alone (control), CHX (control (CHX)), TNF-α plus CHX (A), Fas ligand (FasL: B) or STS (C) for 2 h. Each symbol stands for the means ± S.E.M. (vertical bar) of 10 experiments. $^*P < 0.05$ vs. control cell volume at the corresponding time point.

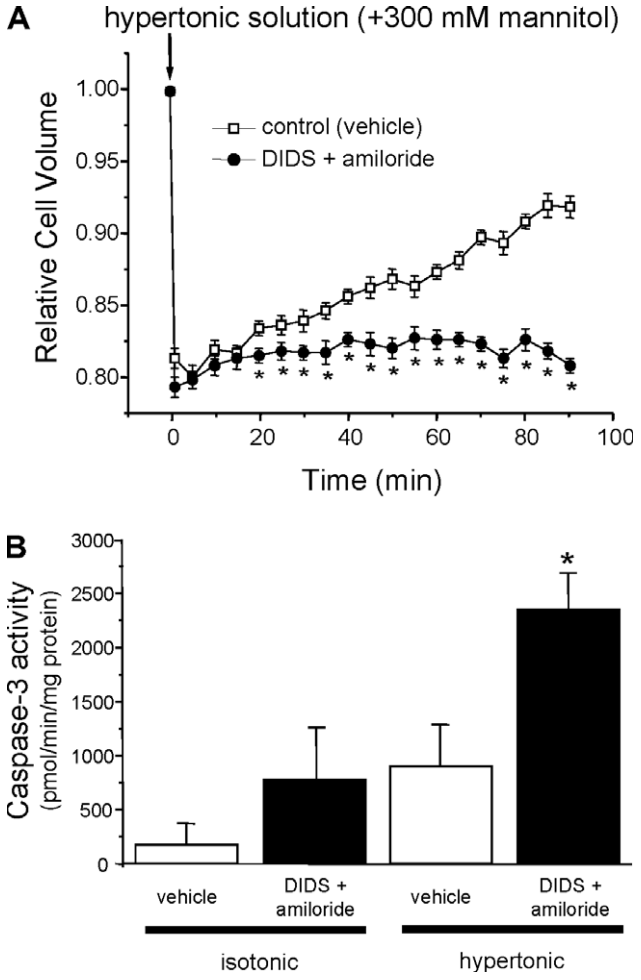

Fig. 2. Effects of combined application of DIDS and amiloride on the RVI (A) and caspase-3 activity (B) in HeLa cells. Each symbol represents the means ± S.E.M. (vertical bar) of 10 experiments. $^{*}P < 0.05$ vs. control (vehicle alone). (A) Cell volume was measured immediately upon application of hypertonic stress (300 → 600 mosmol/kg-H_2O) in the absence (control: open squares) or presence of DIDS + amiloride (filled circles). (B) Caspase-3 activity measured at 6-h after incubation in isotonic (300 mosmol/kg-H_2O) or hypertonic (600 mosmol/kg-H_2O with 300 mM mannitol) solution in the presence of vehicle alone (open column) or together with DIDS and amiloride (filled column).

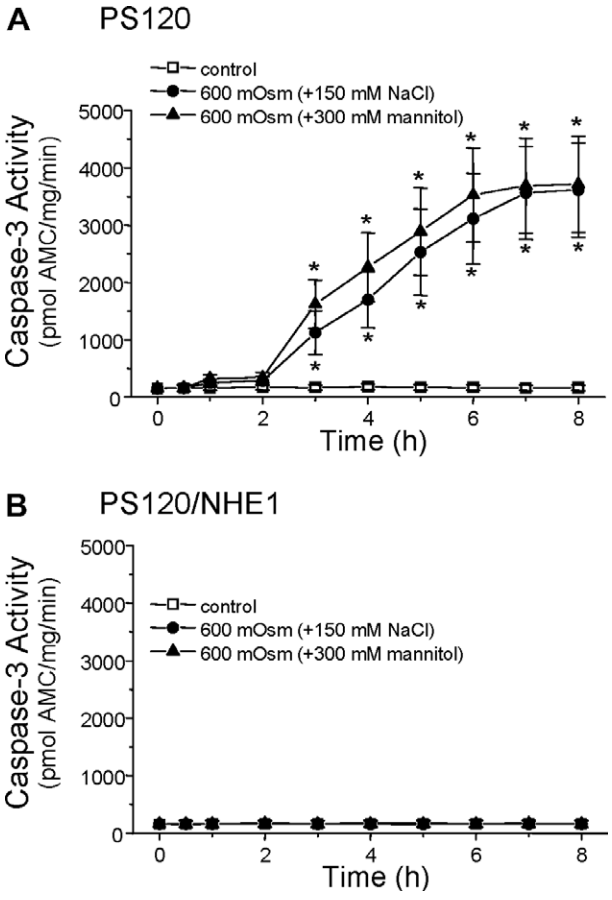

Fig. 3. Effects of hypertonic stress on caspase-3 activity in PS120 cells which lack RVI (A) and PS120/NHE1 cells which exhibit RVI (B). Time courses of changes in caspase-3 activity were observed in PS120 and PS120/NHE1 cells in isotonic solution (control: open squares) or hypertonic solution (600 mosmol//kg-H_2O) containing 150 mM NaCl (filled circles) or 300 mM mannitol (filled triangles). Each symbol represents the means ± S.E.M. (vertical bar) of 10 observations. $^{*}P < 0.05$ vs. control (isotonic) at the corresponding time point.

3.3. Rescue from hypertonicity-induced apoptosis in PS120 cells by restoration of RVI

It was shown that PS120 cells, which are deficient in NHE1 expression [19], fail to exhibit volume regulation after osmotic shrinkage, but that overexpression of NHE1 can restore the ability to undergo RVI [16]. We confirmed that RVI occurs in PS120/NHE1 cells but not in PS120 cells under hypertonic conditions (600 mosmol/kg-H_2O; data not shown). We then examined whether hypertonic stress induces apoptosis in PS120 cells which lack RVI and whether hypertonicity-induced apoptosis is prevented in PS120/NHE1 cells which exhibit RVI.

As shown in Fig. 3, activation of caspase-3 was observed over 2 h after exposure to hypertonic solution prepared with either 150 mM NaCl or 300 mM mannitol in PS120 cells (A) but not in PS120/NHE1 cells (B). Laddering of DNA was also observed after more than 4 h of hypertonic stress in PS120 cells but was never observed in PS120/NHE1 cells (Fig. 4). Exposure to hypertonic solution (containing either 150 mM NaCl or 300 mM mannitol) resulted in the reduction, in a time-dependent manner, of cell viability in PS120 cells, as assessed by the MTT assay. On the other hand, hypertonic stress never induced significant reduction of cell viability in PS120/NHE1 cells, as shown in Fig. 5. These data indicate that conferment of the ability to undergo RVI on PS120 fibroblasts prevented apoptotic cell death under hypertonic conditions.

4. Discussion

Most cell types are known to exhibit volume regulation after osmotic shrinkage by the RVI mechanism [11–14]. In contrast, cell shrinkage persists in cells undergoing apoptosis [7–10]. In the present study, we have, for the first time, demonstrated that the ability to undergo RVI is impaired in human epithelial HeLa cells stimulated with either a death receptor- or mitochondrion-mediated apoptosis inducer (Fig. 1). Thus, there exists a possibility that not only the induction of AVD [9,10], but also the dysfunction of RVI, are essential elements of apoptosis. This hypothesis was suggested by previous observations

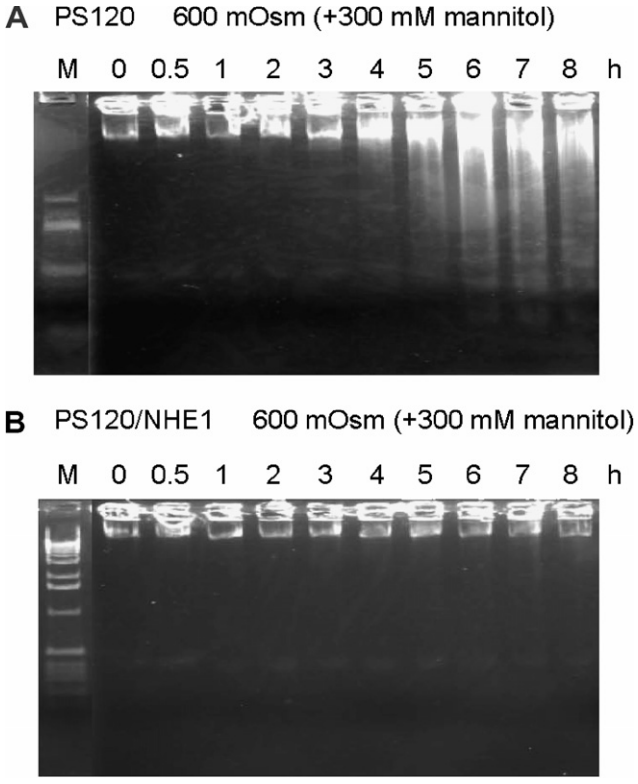

Fig. 4. Effects of hypertonic stress (600 mosmol/kg-H_2O with 300 mM mannitol) on DNA integrity in PS120 cells which lack RVI (A) and PS120/NHE1 cells which exhibit RVI (B). Internucleosomal DNA cleavage (DNA ladder) was observed as early as 5 h after a hypertonic challenge in PS120 cells but never in PS120/NHE1 cells up to 8 h after hypertonic treatment. Data are representative of triplicate experiments. M: 100 bp marker.

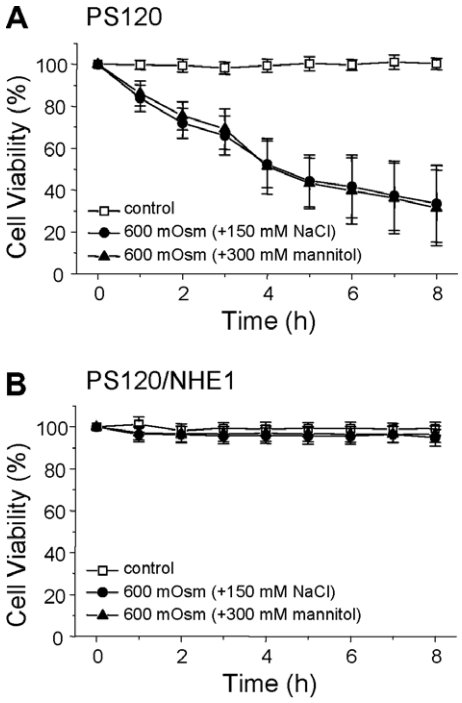

Fig. 5. Effects of hypertonic stress on cell viability in PS120 cells which lack RVI (A) and PS120/NHE1 cells which exhibit RVI (B). Time courses of changes in cell viability were monitored by the MTT assay in PS120 and PS120/NHE1 cells in isotonic solution (control: open squares) or hypertonic solution (600 mosmol/kg-H_2O) containing 150 mM NaCl (filled circles) or 300 mM mannitol (filled triangles). Each symbol represents the means ± S.E.M. (vertical bar) of 10 experiments. $^*P < 0.05$ vs. control (isotonic) at the corresponding time point.

that osmotic cell shrinkage per se leads to apoptosis in cells that lack RVI, but not cells that exhibit RVI [15]. Here, we have substantiated this hypothesis by the following observations: first, even in HeLa cells with the ability to undergo RVI, apoptosis was induced by hypertonic stimulation when the RVI was pharmacologically blocked (Fig. 2); and second, hypertonicity-induced apoptosis was prevented even in NHE1-deficient PS120 fibroblasts, when the ability to undergo RVI was conferred on the cells by transfection with NHE1 (Figs. 3–5).

RVI occurs by uptake of NaCl and osmotically obligated water, and volume-regulatory NaCl influx is mediated by parallel activation of NHE and AE, by operation of NKCC or NCC, and/or by shrinkage-induced activation of a Na^+-permeable cation channel [11–14]. Thus, the dysfunction of RVI associated with apoptosis may be the result of impaired activity of the above transporters or channels. Impaired parallel activation of NHE and AE in apoptotic cells was suggested by the present observation that similar RVI inhibition was induced by application of an NHE blocker, amiloride, and an AE blocker, DIDS (Fig. 2A). Furthermore, our preliminary results showed that the effect of staurosporine and that of combined applications of amiloride and DIDS were not additive (N. Takahashi and Y. Okada, unpublished observations). Above all, inhibition of NHE activity seems to be the most probable component, because the present study showed that restoration of molecular expression of NHE1 not only conferred the ability to undergo RVI on PS120 fibroblasts but also rescued the cells from hypertonicity-induced apoptosis (Figs. 3–5). In fact, it was found that inhibition of an NHE induced apoptosis parallel to that induced by withdrawal of interleukin-2 [20] and by stimulation of Fas [21] in lymphocytes. Also, it was found that NHE1 overexpression largely prevented STS-induced apoptosis in HEK293 cells [22]. Apoptosis-associated inhibition of an NHE might be induced by a mechanism which depends on the activation of Src-like kinase Lck^{56} [21] and/or caspase-3 [22]. In the present study, however, the dysfunction of RVI was observed after a 2-h exposure to apoptotic inducers (Fig. 1), at a time before caspase-3 activation would occur [9]. Since apoptosis is frequently coupled to intracellular acidification [23,24], there is a possibility that the dysfunction of RVI in HeLa cells stimulated with an apoptosis inducer is mediated by intracellular acidification. However, Tafani et al. [25] showed that apoptosis induction with staurosporine increased the intracellular pH (pH_i), whereas apoptosis induction with TNF-α lowered the pH_i value in HeLa cells. Thus, it is likely that the RVI inhibition induced by stimulation with either a death receptor- or mitochondrion-mediated apoptosis inducer is independent of pH changes in HeLa cells.

Hypertonic stimulation is known to stimulate NHE, thereby eliciting significant intracellular alkalinization in HCO_3^--free conditions [16,26–28]. Thus, there exists a possibility that impaired intracellular alkalinization upon hypertonic stimulation is involved in apoptosis induction in HeLa cells treated with an NHE1 blocker (amiloride) together with an AE blocker (DIDS) and in NHE1-deficient PS120 cells. However, the

present experiments were conducted under HCO_3^--containing conditions where pH_i can be controlled by parallel functions of NHE and AE, and may not largely be affected by simultaneous inhibition of NHE and AE. In fact, our preliminary studies with a fluorescent pH_i indicator (BCECF) revealed that the pH_i value observed in the presence of HCO_3^- under hypertonic conditions was not significantly affected by combined applications of amiloride and DIDS in HeLa cells (pH 7.22 ± 0.04 ($n = 7$) and 7.20 ± 0.01 ($n = 5$) without and with amiloride and DIDS, respectively: N. Takahashi, unpublished observations) and by NHE1 transfection in PS120 cells (pH 7.42 ± 0.03 ($n = 6$) and 7.41 ± 0.04 ($n = 6$), respectively: N. Takahashi, unpublished observations). Therefore, it appears that hypertonic stress-induced apoptosis under RVI-lacking conditions is independent of pH_i changes in both HeLa and PS120 cells.

In conclusion, apoptotic stimulation induces inhibition of RVI, at least in part by impairing activity of an NHE. The dysfunction of RVI is essential for the induction of apoptosis, presumably by promoting sustained cell shrinkage.

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