Inhibition of Protein Kinase Akt1 by Apoptosis Signal-regulating Kinase-1 (ASK1) Is Involved in Apoptotic Inhibition of Regulatory Volume Increase*[S]

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Most animal cell types regulate their cell volume after an osmotic volume change. The regulatory volume increase (RVI) occurs through uptake of NaCl and osmotically obliged water after osmotic shrinkage. However, apoptotic cells undergo persistent cell shrinkage without showing signs of RVI. Persistence of the apoptotic volume decrease is a prerequisite to apoptosis induction. We previously demonstrated that volume regulation is inhibited in human epithelial HeLa cells stimulated with the apoptosis inducer. Here, we studied signaling mechanisms underlying the apoptotic inhibition of RVI in HeLa cells. Hyperosmotic stimulation was found to induce phosphorylation of a Ser/Thr protein kinase Akt (protein kinase B). Shrinkage-induced Akt activation was essential for RVI induction because RVI was suppressed by an Akt inhibitor, expression of a dominant negative form of Akt, or small interfering RNA-mediated knockdown of Akt1 (but not Akt2). Staurosporine, tumor necrosis factor-α, or a Fas ligand inhibited both RVI and hypertonicity-induced Akt activation in a manner sensitive to a scavenger for reactive oxygen species (ROS). Any of apoptotic inducers also induced phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) in a ROS-dependent manner. Suppression of (ASK1) expression blocked the effects of apoptosis, in hypertonic conditions, on both RVI induction and Akt activation. Thus, it is concluded that in human epithelial cells, shrinkage-induced activation of Akt1 is involved in the RVI process and that apoptotic inhibition of RVI is caused by inhibition of Akt activation, which results from ROS-mediated activation of ASK1.

Animal cells possess the ability to control their cell volume and use it to confront osmotic stress caused by an imbalance in the osmolarities of the intracellular and extracellular milieu (1). Cell volume regulation after osmotic cell shrinkage is called regulatory volume increase (RVI), and that which occurs after swelling is called regulatory volume decrease. RVI and regulatory volume decrease occur mainly through NaCl uptake and KCl release, respectively, which are mediated by the operation of a number of volume-regulatory ion channels and transporters (1, 2). However, apoptotic cells undergo persistent cell volume reduction. This whole cell shrinkage, termed apoptotic volume decrease (3, 4), continues until cell fragmentation, and the cells do not exhibit RVI. In HeLa cells stimulated with Fas ligand (FasL), tumor necrosis factor-α (TNFα) or staurosporine (STS), we have recently shown that the ability to undergo RVI is impaired even after osmotic shrinkage under hypertonic conditions (5). Thus, it appears that persistent cell shrinkage in apoptotic cells comprises apoptotic volume decrease induction and RVI dysfunction (6). It has been concluded that RVI dysfunction is necessary for hyperosmotic stress-induced apoptosis on the basis of the following observations. First, hypertonicity-induced cell shrinkage led to apoptotic cell death in cells that endogenously lack the ability to undergo RVI but not in RVI-capable cell types such as human epithelial HeLa cells (6, 7). Second, even in HeLa cells, apoptosis was induced by hypertonic stress alone when RVI was inhibited by blocking the activity of volume-regulatory ion transporters (Na+/H+ exchangers (NHE) and anion exchangers) (5) or of volume-regulatory cation channels (hypertonicity-induced cation channels) (8). Furthermore, even under normotonic conditions, HeLa cells were found to undergo apoptosis when persistent cell shrinkage was induced by deprivation of extracellular Cl− (9) or Na+ (10), which impairs RVI by preventing NaCl uptake (6). Moreover, STS-induced apoptosis was found to be restored in HeLa cells when the machinery of RVI was preactivated by hypertonic stimulation prior to application of STS (11). Taken together, these observations suggest that RVI dysfunction is necessary for apoptosis induction in HeLa cells. Thus, to understand apoptosis better, it is important to investigate the intracellular signals coupled to apoptotic inhibition of cell volume regulation in shrunken cells.

So far, a wide variety of intracellular signaling events has been implicated in osmotic cell shrinkage or RVI (1) as well as in apoptotic processes (12–15). However, nothing is known as to...
which signal transduction mechanism is involved in apoptotic inhibition of RVI. The present study was carried out to address this question by focusing on Akt, a Ser/Thr protein kinase alter-
Cells and Cell Volume Measurements—HeLa cells were cultured in 10% fetal bovine serum/minimum essential medium at 37 °C in 5% CO₂/95% air under humidified conditions. Kinase inhibitors were prepared as stock solutions in dimethyl sulfoxide and diluted with experimental buffers just before use. To induce apoptosis, HeLa cells were treated with 4 μM STS, 2 ng/ml TNFα plus 1 μg/ml CHX (TNFα/CHX), or 0.1 mg/ml FasL. For the present experiments, the cells were studied 2 h after stimulation with an apoptosis inducer because reduction in cell viability did not start within 2 h of stimulation with STS, TNFα/CHX, or FasL. (3) or with 100 μM H₂O₂ (data not shown). Transfection of each expression vector was performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. After the transfection, exogenous gene-expressing cells were selected by G418 (Invitrogen) as a negative control siRNA (NC-siRNA).

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—STS, N-acetylcycteine (NAC), catalase, and Mn(III) tetraakis(4-benzoic acid)porphyrin (MnTBAP) were obtained from Sigma. A phosphatidylinositol (PI) ester analog Akt inhibitor, IL-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt Inhibitor), was from Calbiochem. Anti-Fas antibody as FasL and human TNFα were from PeptoTech (London, England). Cycloheximide (CHX) was from Nacalai Tesque (Kyoto, Japan). Anti-phospho-Akt1/2 (Ser473) was from R&D Systems (Minneapolis, MN). Anti-human Akt1/2 (BD Biosciences), anti-human Akt2 (Rockland, Gilbertsville, PA), anti-phospho-ASK1 (Ser83) (Rockland), and anti-human ASK1 antibodies (R&D Systems) were used for immunoblotting.

Construction of Expression Vectors and siRNAs—Human Akt1 cDNA was kindly provided by Dr. Y. Gotoh (University of Tokyo). A pleckstrin homology domain of Akt1 (N-terminal region: amino acids 1–108), which was used as dominant negative Akt (dn-Akt), was amplified by PCR using the cDNA and primers with a restriction enzyme site (forward primer with XhoI site, CGGC-TCGAAGCTATGACCCGACGCTG-CTATTGTA; reverse primer with EcoRI site and STOP codon, CGA-ATTCTCAGTCCTGCTCTTCT-TGAGGCCC). The PCR product was digested with Xhol and EcoRI after checking the sequence and then ligated into Xhol/EcoRI sites of a pDsRed2-C1 plasmid (BD Biosciences Clontech), which was used as a mock vector (Mock). Human Akt1-, Akt2-, and ASK1-specific siRNAs were purchased from Invitrogen as validated Stealth siRNAs. Knockdown efficiencies were examined by immunoblotting. Fluorescein isothiocyanate-labeled short RNA with a random sequence (BlockIT; Invitrogen) was used as a negative control siRNA (NC-siRNA).
After washing the cells to remove the apoptosis inducers, the cells were detached from their plastic substrate and placed in suspension at a density of $6 \times 10^5$ cells/ml. A volume of 1 ml was applied to the cell size analyzer for 20 s to obtain each mean cell size value. Isoosmotic (310 mosmol/kg-H$_2$O) solution was made of serum-free Dulbeccos' modified Eagle's medium containing 20 mM NaHCO$_3$ and 20 mM HEPES/NaOH (pH 7.4). Hypertonic solution (450 mosmol/kg-H$_2$O) was prepared by adding 150 mM mannitol to the isotonic solution.

Immunoblottings—Immunoblottings were performed as described previously (20). Briefly, after incubation in hypertonic solution and/or with various reagents, the cells were scraped into a total volume of 200 $\mu$l of extract buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM sodium vanadate, and protease inhibitor mixture). The cells in the extracts were sonicated with a probe sonicator with two to three bursts of 5–10 s each and centrifuged at 15,000 rpm for 15 min at 4 °C. The cell lysates were mixed 1:1 with SDS-PAGE sample buffer (BioRad), heated at 95 °C for 5 min. Proteins (20 $\mu$g/lane) were subjected to 4–20% gradient SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, blocked with TBS (10 mM Tris-HCl and 150 mM NaCl (pH 7.15)) supplemented with 5% nonfat milk (blocking solution), and exposed to a primary antibody (4 °C, overnight). The membranes were washed with TBS three to four times and incubated with biotin-labeled anti-rabbit/mouse IgG secondary antibody (at room temperature for 1 h). After several washes with TBS containing 0.1% Tween 20 (TTBS), the membranes were incubated with streptavidin-conjugated alkaline phosphatase for 30 min at room temperature. Protein bands were detected after several washes with TTBS by staining with 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium chloride. Densitometric results of more than three independent experiments were evaluated using ImageJ software (National Institutes of Health). The values for phosphoproteins were normalized by the values for total proteins.

**Statistical Analysis**—The data, presented as means ± S.E., were statistically analyzed using analysis of variance followed by
Benferroni’s test and Student’s t test. Differences were considered significant when \( p < 0.05 \).

**RESULTS**

**Hypertonic Challenge Activates Akt, Inducing Recovery of Cell Volume after Osmotic Shrinkage**—In our previous study, PI-3K inhibitors (wortmannin and LY294002) inhibited the induction of RVI in HeLa cells under hypertonic conditions (21). Therefore, in the present study, we first examined whether suppressing the activity of Akt, a downstream target of PI-3K, inhibits the induction of RVI. As shown in Fig. 1, Akt Inhibitor significantly blocked the induction of RVI in HeLa cells exposed to hypertonic conditions (450 mosmol/kg-H₂O). Also, stable expression of the Akt1 pleckstrin homology domain, a dominant negative form of Akt (dn-Akt), but not expression of vector alone (Mock), inhibited the induction of RVI in HeLa cells. RVI was inhibited to 12 and 39% in HeLa cells treated with Akt Inhibitor and transfected with dn-Akt, respectively. Next, we examined whether Akt is activated by a hypertonic challenge in HeLa cells. As shown in Fig. 1, Akt phosphorylation significantly increased 15 min after a hypertonic challenge and reached a plateau level at 30 min. These results suggest that Akt activation induced by a hypertonic challenge is necessary for the induction of RVI in HeLa cells.

To determine which isoform of Akt is involved in the induction of RVI, the effects of knockdown of the Akt isoforms were then studied by transfecting siRNA for either Akt1 or Akt2 because Akt1 and Akt2, but not Akt3, were found to be predominantly expressed in HeLa cells by our preliminary reverse transcription-PCR studies (data not shown). The knockdown efficiencies are shown in an inset panel in Fig. 2. As shown in Fig. 2, siRNA for Akt1 suppressed the induction of RVI under hypertonic conditions, whereas siRNA for Akt2 showed no effect on the induction of RVI. This indicates that Akt1, not Akt2, is involved in the induction of RVI in HeLa cells.

**Apoptotic Stimuli Inhibit Hypertonicity-induced Akt Activation and the Induction of RVI in a Manner Sensitive to ROS Scavengers**—In our previous study, various apoptotic stimuli inhibited the induction of RVI in HeLa cells exposed to hypertonic conditions (5). Therefore, we determined whether the Akt activation induced during hypertonic exposure is prevented by apoptotic stimulation in HeLa cells. As shown in Fig. 3, Akt phosphorylation was suppressed by 2-h pretreatment with STS (4 μM) or TNFα (50 ng/ml) plus CHX (1 μg/ml). Similar suppression of Akt phos-
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phorylation was observed in the case of 2-h pretreatment with FasL (0.1 mg/ml anti-Fas antibody) (Fig. 3, C and D). Also, H$_2$O$_2$ (100 $\mu$M) was found to suppress hypertonicity-induced Akt phosphorylation (Fig. 3, C and D). It is well known that ROS are involved in the apoptosis induced by various apoptotic stimuli (22–24). Therefore, the involvement of ROS in apoptotic inhibition of hypertonicity-induced Akt activation was examined using several ROS scavengers. As shown in Fig. 3, A and B, NAC (1 mg/ml) and MnTBAP (100 $\mu$M) restored the Akt phosphorylation that was observed to be induced by hypertonicity but suppressed by pretreatment with STS and TNF$_\alpha$/CHX, respectively. The suppression of Akt by FasL and H$_2$O$_2$ was also reversed by treatment with NAC (1 mg/ml) and catalase (100 units/ml), respectively (Fig. 3, C and D). These data indicate that apoptotic stimuli inhibit the Akt activation induced by a hypertonic challenge through intracellular ROS production in HeLa cells.

When cell volume was measured in the presence of ROS scavengers, apoptotic inhibition of the RVI induced by hypertonic exposure was found to be attenuated in HeLa cells. As shown in Fig. 4A, NAC restored the RVI that was inhibited by 2-h pretreatment with STS. Inhibition by TNF$_\alpha$/CHX, FasL, and H$_2$O$_2$ of the hypertonicity-induced RVI was also reduced by MnTBAP, NAC, and catalase, respectively (Fig. 4, B–D). All data for the apoptosis inducer only-treated group at 90 min after hypertonic stimulation are significantly different ($p < 0.05$) from the control data and from the data for apoptosis inducer plus ROS scavenger groups (Fig. 4). These results indicate that in HeLa cells, ROS are key regulatory molecules in the inhibition by various apoptotic stimuli of hypertonicity-induced RVI.

Apoptotic Stimuli Activate ASK1, Inhibiting Hypertonicity-induced Akt Activation and RVI Induction—ASK1 is known to be activated directly by intracellular ROS during apoptotic stimulation (25–27). In fact, treatment with STS or TNF$_\alpha$/CHX for 2 h was found to induce phosphorylation of ASK1 in HeLa cells in a manner sensitive to a ROS scavenger, NAC or MnTBAP, as shown in Fig. 5, A and B. FasL and H$_2$O$_2$ also induced phosphorylation of ASK1 which was sensitive to NAC and catalase, respectively (Fig. 5, C and D).

To elucidate the roles of ASK1 activity in the apoptotic inhibition of RVI, the effects of down-regulation of ASK1 activity by ASK1-specific siRNA were then examined. The siRNA-mediated knockdown of ASK1, the efficacy of which was confirmed by immunoblotting (Fig. 6A, Inset), largely restored RVI even after 2-h pretreatment with STS or TNF$_\alpha$/CHX (Fig. 6).

Finally, an involvement of ASK1 in the apoptotic inhibition of hypertonicity-induced Akt activation in HeLa cells was studied. As shown in Fig. 7, A–C, the inhibition of hypertonicity-induced Akt phosphorylation by STS or TNF$_\alpha$/CHX was prevented in HeLa cells transfected with ASK1-specific siRNA, whereas in NC-siRNA-transfected HeLa cells, the inhibition of Akt phosphorylation still occurred. As shown in Fig. 7, D–F, stable expression of dn-ASK1, which has a point mutation in the catalytic site of ASK1 (28), was also found to prevent inhibition by TNF$_\alpha$/CHX or H$_2$O$_2$ of Akt phosphorylation. These results indicate that in HeLa cells ROS-mediated ASK1 activation under apoptotic conditions is involved in the inhibition of Akt1 and that the apoptotic inhibition of the RVI induction is due to the inhibition of the hypertonicity-induced Akt activation.

DISCUSSION

Apoptosis is essential for maintenance of somatic cell turnover and tissue homeostasis as well as for embryogenesis and organ development under physiological conditions (29–31). Apoptosis is also intimately involved in the pathogenesis of degenerative diseases, autoimmune disorders, cancer, viral infections, and AIDS (32–35). Apoptotic processes are charac-
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FIGURE 7. Reversal of apoptotic inhibition of hypertonicity-induced Akt activation by ASK1-specific siRNA and dn-ASK1. A and B, representative immunoblots, using whole cell lysates, of P-Akt1/2 and total Akt1/2 before (Iso) and 30 min after hypertonic stimulation (450 mosmol/kg-H2O) following 2-h pretreatment with 4 μM STS (A) or 50 ng/ml TNFα plus 1 μg/ml CHX (B) of HeLa cells transfected with NC-siRNA or ASK1-siRNA. C, suppressive effects of siRNA-mediated down-regulation of ASK1 on apoptotic inhibition of hypertonicity-induced Akt activation. Values shown are ratios of P-Akt1/2 to total Akt1/2 levels determined by densitometry and then normalized to the value before hypertonic stimulation (Iso). D and E, representative immunoblots, using whole cell lysates, of P-Akt1/2 and total Akt1/2 before (Iso) and 30 min after hypertonic stimulation (450 mosmol/kg-H2O) following 2-h pretreatment with 50 ng/ml TNFα plus 1 μg/ml CHX (D) or 100 μM H2O2 (E) of HeLa cells transfected with dn-ASK1 and expression vector alone (Mock). F, suppressive effects of dn-ASK1-induced down-regulation of ASK1 on apoptotic inhibition of hypertonicity-induced Akt activation. The values shown are ratios of P-Akt1/2 to total Akt1/2 levels determined by densitometry and then normalized to the value before hypertonic stimulation (Iso). Data represent the means ± S.E. (error bars) of three independent experiments. *p < 0.05 for the hypertonic data in NC-siRNA-transfected or Mock-transfected groups treated with apoptosis inducers versus that not treated with an apoptosis inducer. **p < 0.05 for the hypertonic data in apoptosis inducer-treated NC-siRNA-transfected or Mock-transfected groups versus apoptosis inducer-treated ASK1-siRNA-transfected or dn-ASK1-transfected groups. There is no significant difference between the data for ASK1-siRNA-transfected or dn-ASK1-transfected cells treated with apoptosis inducers and the cells not treated with them.

Akt, also called protein kinase B, is a Ser/Thr protein kinase that is a downstream target of PI-3K that protects cells against apoptosis (39, 40). There are at least three isoforms of Akt (39, 41); Akt1 and Akt2 are ubiquitously expressed, but Akt3 expression is more restricted to neuronal tissues and testis (42, 43). Akt endogenously expressed in Madin-Darby canine kidney cells has been observed to be phosphorylated/activated under hypertonic stress (16), whereas Akt heterologously expressed has been reported to be activated (44) or inactivated (45). In the present study, Akt1 and Akt2 were found to be endogenously expressed in human epithelial HeLa cells, and Akt1 (but not Akt2) was shown to be activated by a hypertonic challenge and to induce RVI. The PI-3K/Akt pathway is known to be an antiapoptotic signal (40, 46–50). A mitochondrion-mediated apoptosis inducer, STS, was reported to reduce Akt phosphorylation in Akt-transfected HEK293 cells (51) and Madin-Darby canine kidney cells (17). In the present study, STS, H2O2, and death receptor-mediated apoptosis inducers TNFα and FasL were all found to inhibit, in a manner dependent on ROS, hypertonicity-induced phosphorylation of Akt endogenously expressed in HeLa cells (see supplemental Fig. S1).

After osmotic shrinkage, RVI occurs through uptake of NaCl and osmotically obliged water. The present study demonstrates that hypertonicity-induced activation of PI-3K/Akt1 is involved in the regulatory volume decrease process (supplemental Fig. S1). Because the RVI mechanism is known to involve activation of hypertonicity-induced cation channels, Na+-K+-2Cl− cotransporter and/or parallel operation of NHE and anion exchanger (1, 2, 52), some of these volume-regulatory Na+ and Cl− channels/transporters might represent downstream effectors of Akt1 or some other signals activated by Akt1 (see supplemental Fig. S1). However, hypertonicity-induced cation channels can be excluded as a candidate because PI-3K inhibitors (woertmann and LY294002) suppressed RVI without significantly affecting the hypertonicity-induced cation channel current in HeLa cells (21). Na+–K+–2Cl− cotransporter is also unlikely to be an effector of Akt1 because RVI was insensitive to an Na+-K+–2Cl− cotransporter blocker bumetanide (100 μM) in HeLa cells.3 By sequence analysis, we found that several consensus sequences for Akt-dependent phosphorylation (RXRXXT/S) exist in the C-terminal region of NHE1. Actually, Akt was reported to phosphorylate NHE1 directly (53). Moreover, RVI was sensitive to an NHE

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blocker amiloride (100 µM) in HeLa cells, suggesting that the parallel operation of NHE and anion exchanger is largely involved in RVI in HeLa cells. Thus, it is possible that NHE represents a downstream effector of Akt1 in HeLa cells. To identify the precise effector of Akt1, however, further experiments are required.

The present study demonstrated that ASK1 was activated by all of these apoptosis inducers in HeLa cells. This is in good agreement with the known fact that ASK1 is a member of the MAPKKK family preferentially activated by various cytotoxic stresses (19, 27) and plays a causal role in cell death induced by a number of stimuli (18). Activation of ASK1 has been shown to be often mediated by ROS (19, 27, 55). The present study also demonstrated that ASK1 phosphorylation induced by STS, TNFα, and FasL was dependent on ROS. A new finding in the present study is that apoptotic activation of ASK1 causes inhibition of shrinkage-induced activation/phosphorylation of Akt1. It is noted that this finding is in contrast to a well known fact that ASK1 ordinarily lies downstream of Akt.

The precise mechanism by which ASK1 inhibits the activation of Akt1 by hypertonicity is not known. However, it is plausible that some MAPKKK/MAPK downstream of the MAPKKK ASK1 is involved therein (see supplemental Fig. S1). Because ASK1 is known to be required for ROS-induced c-Jun N-terminal kinase and p38 activation (56), it is possible that ASK1-induced inhibition of hypertonicity-induced Akt1 activation is mediated by c-Jun N-terminal kinase and/or p38, which are known to be activated by hypertonic stress in many cell types (1, 57). Also, it is noted that inhibition of PI-3K/Akt by insulin receptor substrate-1 (58).

The findings in the present study are summarized as follows (see supplemental Fig. S1): First, phosphorylation/activation of Akt1 (but not Akt2) is induced by hypertonic stimulation and is involved in RVI induction in HeLa cells. Second, both mitochondrion- and death receptor-mediated apoptosis-stimulating agents induce phosphorylation/activation of ASK1 in a manner dependent on ROS. Third, ASK1 thus activated inhibits shrinkage-induced activation of Akt1 thereby inhibiting RVI. These findings may account for why apoptotic cells undergo the persistent shrinkage in apoptotic volume decrease without exhibiting RVI.

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